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Archaeopteryx : ancient bird

THE MORPHOGENESIS OF FEATHER PRIMORDIA IN CHICKEN SKIN

by

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TO MY PARENTS

and to

JOHN and JAN

VOLUME I

Abstract

This thesis examines aspects of two broad problems: first, what governs the time and position at which organs form; second, how do cells build organ primordia? These problems have been investigated in six-day chicken dorsal skin.

To facilitate microscopic observation, skin was cultured on transparent collagen.

Regarding the first problem, patterns formed by feathers in cultured skin are determined, in part, by interaction between adjacent sites shortly before primordium morphogenesis. Histology of fresh skin confirmed that arrays of dermal cells extend from morphologically distinct sites towards new ones, but cutting skin before morphogenesis did not prevent normal pattern development; thus extension of cell patterns does not define the feather pattern. Fused primordia sometimes formed in culture, suggesting that primordia are not spaced by steric interaction. Thus spacing appears not to depend on dermal morphogenesis.

Pre-existing organisation also influences the pattern. Development of cut skin showed that the time of morphogenesis is preset over most of the dorsal skin before any primordia form. The actual pattern differs, at morphogenesis, from a simple packing pattern: this, and other evidence, suggests that pre-existing dermal organisation helps determine primordium position.

Regarding the second problem, the development of condensed dermis in primordia was studied. Quantitative analysis showed that proliferation cannot account for the increase in cell numbers in condensations in culture. Histology of fresh skin, and filming, suggested that cells do not migrate into condensations along

arrays. Short-range centripetal movement is suggested. Experiments using colcemid showed that neither proliferation nor migration are essential for changes in primordium shape up to the feather-bud stage. Dissection experiments showed that dermal cells adhere more strongly to the basement membrane in primordia than elsewhere. These results suggest that changes in cell properties (possibly adhesiveness and contractility) at the primordium site are responsible for condensation morphogenesis and stability.

Similarities with other organ primordia are emphasised.

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CONTENTS

VOLUME I

		Page
CHAPTER 1	**INTRODUCTION**	
1.	GENERAL INTRODUCTION	1
2.	THE FEATHER PATTERN	
2.1	General Description	4
2.2	Feather, Scale, and Hair Patterns	6
2.3	The Site of Pattern Determination	11
2.4	Progressive Stages in Pattern Determination	16
2.5	Contemporary Models of Feather Pattern Formation	30
2.6	Interim Conclusions	43
3.	THE DEVELOPMENT OF INDIVIDUAL FEATHER PRIMORDIA	
3.1	Processes Leading up to Primordium Formation	44
3.2	Necessary Tissue Interactions	50
3.3	The Possible Rôle of Dermal Morphogenesis in Pattern Determination	55
3.4	The Early Stages of Primordium Development	63
3.5	Conclusions	81
4.	LINES OF FURTHER INVESTIGATION	82
CHAPTER 2	**GENERAL MATERIALS AND METHODS**	
1.	MATERIALS	
1.1	Eggs	85
1.2	Solutions	85
2.	METHODS	
2.1	Dissection	87
2.2	Organ Culture	89
2.3	Culture of Skin Fragments on Aligned Collagen Substrata	91
2.4	Observation of Living Material	91
2.5	Observation of Fixed Material	96
CHAPTER 3	**COMPARISON OF SKIN DEVELOPMENT IN CULTURE WITH SKIN DEVELOPMENT IN VIVO**	
1.	INTRODUCTION	100
2.	METHOD	100
3.	OBSERVATIONS	
3.1	Note on the Correlation of Observations Made by Dissecting Microscopy and Histology	101
3.2	Observations on Living Material	103
3.3	Observations on Fixed Material	112
4.	DISCUSSION	117

CONTENTS (contd)

Page

CHAPTER 4 THE TIME AND POSITION OF PRIMORDIUM MORPHOGENESIS

1. INTRODUCTION 124

2. OBSERVATIONS ON FRESHLY EXCISED SKIN
   - 2.1 General Morphology of the Emerging Feather Pattern 124
   - 2.2 Extent of Dermal Cell Patterns Lateral to Existing Primordia 125

3. OBSERVATIONS ON CULTURED SKIN
   - 3.1 General Morphology of the Pattern 129
   - 3.2 Experiments on Pattern Formation in Culture 133

4. DISCUSSION
   - 4.1 The Relation of Dermal Cell Patterns to the Emerging Feather Pattern 145
   - 4.2 The Initiation of Development in Successive Rows of Primordia 148
   - 4.3 The Positions of New Primordia 150

VOLUME II

CHAPTER 5 THE DYNAMICS OF DERMAL MORPHOGENESIS

1. INTRODUCTION 156

2. DERMAL MORPHOGENESIS IN VIVO
   - 2.1 Introduction 156
   - 2.2 Observations 156
   - 2.3 Discussion 163

3. QUANTITATIVE ANALYSIS OF DERMAL CONDENSATION IN CULTURE
   - 3.1 Introduction 169
   - 3.2 Method and Calculations 170
   - 3.3 Results 177
   - 3.4 Discussion 200

4. TIME-LAPSE FILMING OF CELL MOVEMENT IN THE DERMIS 217

5. EXPERIMENTS USING COLCEMID
   - 5.1 Introduction 219
   - 5.2 Observations on the Effect of Colcemid on Primordium Development 221
   - 5.3 Evidence Relating to the Nature of Cell Movement in Colcemid-treated Dermis 231
   - 5.4 Discussion 238

6. EVIDENCE FROM DISSECTION EXPERIMENTS
   - 6.1 Introduction 242
   - 6.2 Method 243
   - 6.3 Results 244
   - 6.4 Discussion 260

7. GENERAL DISCUSSION 264

CONTENTS (contd)

Page

CHAPTER 6 GENERAL DISCUSSION

1. TEMPORAL CONTROL OF MORPHOGENESIS 277

2. THE POSITIONS OF FEATHER PRIMORDIA
   - 2.1 Introduction 283
   - 2.2 Anterio-Posterior Position of Initial Primordia and Chevrons 283
   - 2.3 Local Interactions Determining Primordium Position 297

3. A PERSPECTIVE VIEW OF FEATHER PRIMORDIUM DEVELOPMENT
   - 3.1 Introduction 300
   - 3.2 Mechanisms of Morphogenesis 300
   - 3.3 The Rôle of Close Apposition in Cell and Tissue Interactions 309
   - 3.4 Evolutionary Relationships 311

4. CONCLUSIONS 315

REFERENCES

CHAPTER 1

INTRODUCTION

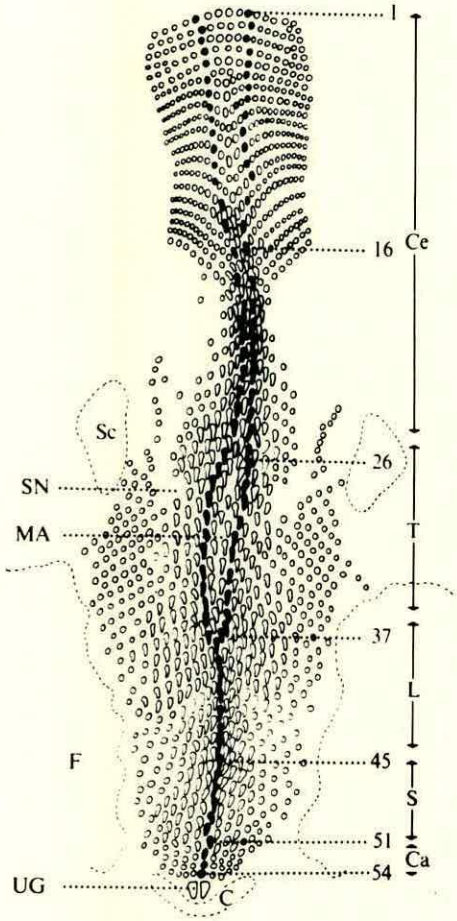


Fig. 1.2 Schematic diagram of feather positions in the spinal pteryla of an 11 - 12 day old chicken embryo (Stage 36).

Black primordia form the initial row. Numbers refer to the number of initial primordia counting from the anterior end of the cervical tract. Tracts: Ce cervical, T thoracic, L lumbar, S sacral, C caudal. Features: SN scapular notch, AM mid-dorsal apertium, UG Uropygeal glands. Other pterylae: Sc scapular, F femoral, C caudal. (From Sengel, 1976b.)

1. GENERAL INTRODUCTION

This thesis is concerned with the earliest morphological stage of feather development, the formation of the feather primordium.

Feather primordia begin their development during the period between six and nine days after the egg is laid. They appear to the naked eye as low domes about 0.24 mm in diameter on the skin surface. Both main components of the skin - dermis and epidermis - play a part in the formation of primordia (Wessells, 1965; Ede, Hinchliffe, and Mees, 1971) (Fig. 1.1). The dermis is a mesenchymal

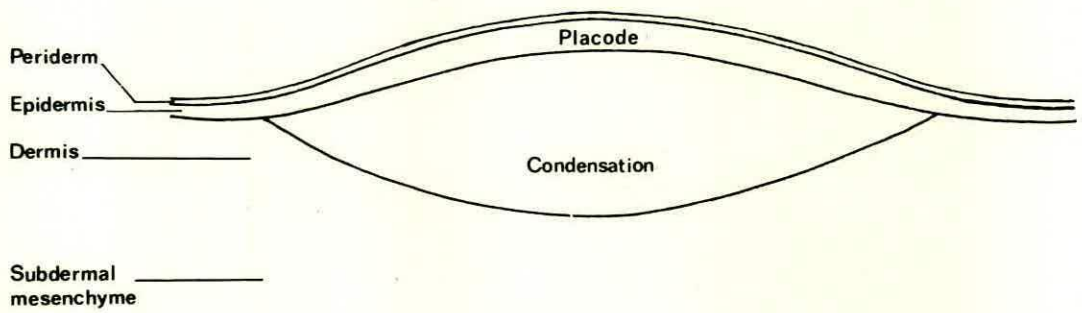


Fig. 1.1 Diagram to show the general arrangement of tissues in the forming feather primordium

tissue composed of stellate and spindle-shaped cells in mutual contact over only a small proportion of their surface; a substantial part of the tissue volume is extracellular space, filled with fluid and a chemically complex meshwork of fibres: in the primordium,

dermal cells become rounded and closely apposed to form a condensation. The dermis is overlain by a two-layered epithelial tissue. This comprises a superficial, thin sheet of cells - the periderm - beneath which lies the true epidermis which is a sheet of columnar cells: the epidermal cells elongate, in the primordium, to form a thick placode. Feather primordia form the serially repeated units of a highly regular spatial pattern over the surface of the skin (Fig. 1.2). This pattern emerges in a very ordered way: an initial row forms in a predictable position and subsequent rows are added sequentially at regular time intervals. Each primordium grows out from the skin surface to form a knob-like structure, the feather-bud. This in turn, elongates into a complex, multicellular filament upon which barb ridges and the elaborate products of epidermal differentiation develop to form the first generation, down feather. Attention is confined here to the initial development of the primordium, up to the beginning of the feather-bud stage. A review of the subsequent development of the feather has been given by Lucas and Stettenheim (1972).

The development of feather primordia presents two major problems. First, what determines the time and position at which organ development will occur? This is essentially a problem of pattern formation over the surface of the skin. Second, what kind of cell behaviour is responsible for shaping the distribution and morphology of cells as the new organ primordium is constructed. This latter problem is concerned with the morphogenesis - that is, the creation of form - of individual primordia.

This developmental system is potentially an excellent model in which to study the general problems of pattern formation and morphogenesis. The main advantage it offers is the regularity of

its temporal and spatial development. On one hand, pattern development is highly predictable, and is thus well suited for experimental analysis. On the other hand, the dynamics of morphogenesis can be readily studied, for a regular sequence of stages is present in a single piece of skin.

Feather development has interested biologists since the 17th Century when Malpighi made the first recorded study (Adelmann, 1966; quoted by Lucas and Stettenheim, 1972). By the end of the 19th Century, the histological structure and development of primordia had been beautifully drawn and described (reviewed by Davies, 1889). However, in the first part of the present century interest centred on the development, symmetry, and regional differences of the adult feather (Fraps and Juhn, 1936; Lillie, 1942; Wang, 1943), and it is only in the last 25 years that the earliest processes of feather formation have received experimental attention. During this period, the dominating approach has been a long and continuing analysis of tissue interactions in primordium development (review by Rawles, 1965; Kratochwil, 1972; Sengel, 1976a) which has given useful, but limited, information regarding the problems posed above. Certainly, there have been several experimental studies of pattern formation (Linsenmayer, 1972; reviewed by Sengel, 1975) and morphogenesis (Wessells, 1965; Stuart, Garber, and Moscona, 1972), but the problems remain unresolved. Moreover, relatively little consideration has been given to aspects of feather development which might shed light on the development of other organ primordia. Instead, attention has focussed on features of development which are unusual, or particular to the feather system; for example, on the observation that successive rows of primordia form in an initially closely packed

pattern (Sengel, 1976b) or that primordia develop in association with unique cell patterns in the dermis (Stuart et al, 1972).

Evidence relating to the formation of the feather pattern and the morphogenesis of individual primordia is re-examined in the following two sections of this chapter.

2. THE FEATHER PATTERN

This section examines the evidence regarding processes which determine the feather pattern. As background, it is first necessary to describe the feather pattern and to look at its relation to patterns formed by other appendages. Evidence indicating the importance of the dermis in determining the time and position of primordium development is then reviewed. The remaining two discussive parts of the section deal with the crucial question of how the dermis is organised with respect to pattern determination. The first discusses the contribution of organisation progressively established in the prospective dermal tissues up to the time when primordia form. The second reviews published models which seek to explain the determination of the feather pattern at the time of morphogenesis.

2.1 General Description

Only certain areas of the skin - pterylae - develop a dense pattern of feathers: between these lie apertia in which few, if any, feathers form. The large pterylae may be subdivided into tracts, according to their location and differences in pattern (Lucas and Stettenheim, 1972). Within these areas the loci of almost all

the principal feathers of the bird are established during the initial stages of skin development (Holmes, 1935). These same loci form several generations of feathers, including the down feathers, the juvenile contour feathers, and the adult contour feathers. In the chick, at around 13 days of development, after the principal sites have formed, 'hair feathers' are added, usually associated in ones and twos with the principal sites.

That all the pterylae have similar temporal and spatial patterns of development was first shown by Holmes (1935), and later confirmed in more detail for the humeral, thigh, and spinal pterylae (Saunders and Gasseling, 1957; Linsenmayer, 1972; Mauger and Sengel, 1970; Ede, Hinchliffe, and Mees, 1971). The lumbo-sacral tract of the spinal pteryla provides the best studied example. In morphological and histochemical accounts of the developing skin (described in Section 3), there have been no reports of periodic pattern in the skin before the first primordia begin to form in the pteryla. The first three or four primordia form along the midline in the posterior lumbar region (Sengel, 1971; Ede et al, 1971; Stuart et al, 1972) at Stage 29 to 30 (all stages indicated refer to the standard series of Hamburger and Hamilton (1951)). This row extends in both directions by the addition of successive, closely spaced primordia, while in the anterior lumbar region, where the medial area remains bare, two initial rows of primordia form, one on either side of the midline (See Fig. 1.2). Successive rows are added parallel and lateral to these initial rows at approximately six-hour intervals (Hamburger and Hamilton, 1951). Each new primordium lies between, and close to, two neighbours in the preceeding row so that a characteristic 'diamond' pattern emerges (Fig.1.2).

Sengel (1976b) reported that this basic pattern is modified as the skin grows: anterio-posterior exceeds lateral growth so that the separation between primordia in the same row exceeds the separation between adjacent rows. This gives the appearance of diagonal rows - chevrons - lying across the pteryla. In most pterylae these are curved.

Though the same diamond pattern may be recognised in every pteryla, each tract in the 10 day-old embryo is characterised by the size of primordia, their spacing, the number of primordia in the initial row, the number of lateral rows, and the curvature of the chevrons (Sengel, 1976b). These regional characteristics of the embryonic pattern remain in the adult patterns (Lucas and Stettenheim, 1972). In addition, adult feathers have tract-specific structural features which show graded variations across the tract (Fraps and Juhn, 1936; Lucas and Stettenheim, 1972). Feather patterns and structure vary in detail between species (Lucas and Stettenheim, 1972; Sengel, 1975).

2.2 Feather, Scale, and Hair Patterns

The feather is one of a series of evolutionarily related skin appendages and, before proceeding to consider the mechanisms of feather pattern formation, we should know whether we are dealing with a unique system or one of a set from which we can draw pertinent comparisons. How does the feather pattern compare with those of scales and hairs? Are there any indications of basic similarities between the mechanisms responsible for the patterns of these appendages? Aspects of the developmental relations between these appendages have previously been reviewed by Maderson (1965), Dhouailly (1975), and Sengel (1976b).

Scale patterns

The scale is the closest evolutionary relative of the feather. Palaentological evidence indicates that birds evolved from the Triassic Reptilian group, the Thecodonta, (Heilmann, 1926; Romer, 1966; Walker, 1972), and it is reasonable to assume that feathers evolved from the horny scales (corneoscutes) of these reptiles, though the selective forces and sequence of evolution are widely disputed (Lucas and Stettenheim, 1972; Maderson, 1972; Regal, 1975).

The lizard, Lacerta muralis, provides the best example of a Reptilian corneoscute pattern which has been studied experimentally (Dhouailly, 1975). The dorsum comprises small, polygonal, non-overlapping scales of quite regular size and spacing in a diamond array, though this is less regular than the feather pattern. The ventrum is regularly patterned with large, overlapping scales elongated medio-laterally and lying in three anterio-posterior rows on either side of the midline.

Of Avian scales, those of the tarsometatarsus provide the best-studied example. These scales are large, overlapping, and elongated transversely across the superior surface of the foot (Sawyer, 1972a). Their well-defined patterns show consistent differences between species, for example, between chick and duck (Sengel, 1971). Scales of the plantar pad, in contrast to tarsometatarsal scales, are small, polygonal, and non-overlapping and, though they show a definite range of sizes and spacing, the overall pattern is irregular (Linsenmayer, 1972). Both types of scale develop in a consistent time sequence across the skin.

Hair patterns

Like birds, mammals evolved from Reptilian stock (Romer, 1966), and it is generally thought that hairs probably evolved from

parts of, or in close association with, corneoscutes (Romer, 1949; Spearmann, 1964).

Pelage hairs of the coat form an irregular pattern, though they show a particular range of spacing (Claxton, 1964). Their development is reviewed by Sengel (1976b). From the beginning of development, the follicles are not closely packed. The first set of follicles (the central primaries) forms in a series of phases. At each new phase, follicles form with a well-defined range of distances from existing ones: between phases, the follicles become separated as the skin grows. Following this phasic development, two smaller, lateral primary follicles are formed equidistant from each central primary. The resulting trios form the basis of the adult pattern. Around each trio are finally added a number of secondary follicles.

Many mammals also produce whiskers (vibrissae) on the upper lip. These large hairs form a distinct pattern. For example, in the mouse a particular number develop in short, dorso-ventral rows which form in a regular sequence, the first forming near the eye and the last near the nasal pit (Dhouailly, 1973).

These patterns may be grouped into two classes: irregular and regular patterns. In the former class, the distance between primordia may vary within a certain characteristic range (Claxton, 1964), but there is otherwise no regular or consistent spatial relation between them. In the latter class, the patterns are consistent and regular with respect to the spacing of primordia and with respect to the positional relation of primordia to one another and to adjacent embryonic structures. Similarities in the development of patterns within the same class may reflect similarities in

the underlying mechanisms of pattern determination: in some cases irregular patterns add primordia as the skin grows, but regular patterns generally emerge in a strict time sequence, morphogenesis begins at a well-defined position (usually near the boundary of the pattern) and spreads across the skin. Moreover, the fact that the same type of appendage forms regular and irregular patterns in adjacent areas of the same animal (for example, vibrissae and pelage hairs) probably reflects basic similarities in the formation of these two classes of pattern. However, this descriptive evidence is circumstantial. Experimental analysis provides firmer evidence that the constraints determining the patterned morphogenesis of different appendages are essentially similar.

Experimental Studies

The development of all cutaneous appendages depends crucially on interactions between dermis and epidermis (Kollar, 1972; Sengel, 1976a). Sensitive comparisons between developmental programmes for different patterns and appendages can thus be made by assaying the capacity of tissue which normally forms one type of appendage to participate in the formation of another. A limitation of this approach is that the nature of these interactions is not known, so that such comparisons are, at present, empirical. The method (detailed by Rawles (1963)) is to dissociate the dermis from the epidermis before morphogenesis begins, using a mild trypsin treatment, and to combine the respective tissues from different sources. The combination is cultured - usually on the chorioallantoic membrane (CAM) of a host chicken - and the developmental outcome is observed.

The simplest and most striking indication of the interrelation of feather and scale patterns is the formation of composite

structures of the two appendages in scale-type patterns. These composite structures occur in certain experimental combinations and, more rarely, in untreated chickens (Rawles, 1963). For example, when leg mesoderm and wing ectoderm (the respective sources of scale-forming dermis and feather-forming epidermis) were taken from 3 to $3\frac{1}{2}$ day-old chickens and grafted, in combination, to the wing stump of a chicken host the resulting limb formed a foot which bore feather filaments alone, or in association with scales (Sengel and Pautou, 1969). According to Sengel (1976b) these filaments were arranged in a scale-type pattern and were large or small according to whether they form$^{ed}_{\Lambda}$ in areas of the foot normally populated by large or small scales (Sengel, 1976b).

Similar comparisons have been made between Avian, Reptilian, and Mammalian appendage-forming tissues. The evidence points to the same general conclusion: the pattern-forming mechanism which is normally responsible for one appendage pattern is able to direct the patterned morphogenesis of other appendages. For example, the same appendage can form in an irregular or regular pattern depending on the regional origin of the dermis: chicken dorsal epidermis combined with mouse dorsal dermis forms abortive large and small feather-buds arranged like large central and smaller lateral hair follicles of the pelage pattern, while chicken dorsal epidermis combined with mouse upper lip dermis forms large feather-buds arranged in a regular vibrissa-type pattern, (Dhouailly, 1973). Moreover, the pattern-forming organisation for different appendages shows several basic similarities: in the combinations tested the dermis governs the size, shape, and distribution of appendages while the type of appendage or abortive primordium is generally, but not always, governed by the epidermis. Since the results have recently

been reviewed in detail by Sengel (1976a, and b) and Sengel and Dhouailly (1977), they will not be discussed further here.

The experimental evidence thus strongly suggests that the nature of the organisation which initiates morphogenesis in a particular pattern is the same for all skin appendages examined. The evidence does not, however, bear directly on the similarity of the mechanisms responsible for determining the pattern of this organisation. An hypothetical example will make this clear: the pattern of morphogenesis might be initiated by a latent pattern in the form of the concentration of some chemical substance which was capable of initiating the development of any skin appendage, but the precise way in which this chemical pattern itself was defined might be different in different systems. It seems very unlikely, however, that mechanisms determining the pattern of a similar kind of organisation are <u>essentially</u> different. Of course, additional constraints and quantitative differences are presumably responsible for the specific characteristics of the patterns observed.

In conclusion, the evolutionary, descriptive, and experimental evidence combine to strongly suggest that the different skin appendage patterns are determined by essentially the same mechanism.

2.3 <u>The Site of Pattern-Determination</u>

Obvious differences in the origin and structure of the dermis and epidermis and the ease with which these two tissues can be separated have led naturally to the question of which tissue is responsible for defining the feather pattern?

An answer has been approached experimentally by combining these respective tissues from two types of skin whose patterns can be

distinguished, and observing which pattern is expressed. This type of approach carries certain difficulties. Strictly, we cannot be certain that tissue combinations reveal the true balance of pattern-determining influences in intact skin. This problem is particularly serious where the tissues differ markedly in age or developmental state at the time of combination: one tissue may simply be beyond the stage when it could play a part in determining the pattern. Moreover, one tissue may be capable of greater developmental plasticity than the other (Wessells, 1968; Kratochwil, 1972), but this need not indicate that it is normally passive. These objections apply more forcibly to some pieces of evidence than to others, and particularly serious objections will be noted below. Despite these difficulties the evidence points uniformly in one direction: before the beginning of morphogenesis the dermis dominates the distribution of appendages in every experimental situation examined. These pattern-determining properties are present in the dermis from early in development till close to the time of morphogenesis.

Making use of the regional pattern differences in the spinal pteryla, Mauger (1972b) showed that at 2 to 2½ days the somitic mesoderm (which is the source of dorsal dermal cells) carries the primary determinants of the regional differences in pattern. Keiny (Keiny, 1971, quoted by Sengel, 1976b) and Mauger (1972a) showed, using a similar approach, that the somatic lateral plate mesoderm (which is the source of wing and leg dermis) carries the primary determinants of the regional characteristics of the feather pattern in these areas. Cairns and Saunders (1954), examining intermediate stages of development, showed that the regional characteristics of feathers and the distribution of

feathered areas on the thigh are determined by properties existing in the mesoderm of $3\frac{1}{2}$ to 4 day-old embryos.

Two and one-half days before morphogenesis begins, the integument of the thigh already possess properties which enable it to eventually form a regular feather pattern independent of any local interactions with underlying tissues. This was shown by Linsenmayer (1972) who obtained a regular pattern in pieces of skin excised from $4\frac{1}{2}$ day-old embryos and cultured on the CAM. The general formation of feathers in the initial row (Wessells, 1965) and - given an initial row which had formed in vivo - the subsequent development of a diamond pattern (Novel, 1973) are intrinsic properties of lumbar skin isolated close to time of morphogenesis. It has not, however, been established that initial lumbar primordia form in explanted skin in the same positions as they occupy in vivo where they each overlie one element of the axial skeleton (see Section 2.4). This point is relevant to whether, or not, the underlying tissues play a part in determining the pattern in this region.

As regards which tissue of the skin governs the pattern close to the time of morphogenesis, the evidence relating specifically to the feather system is not strictly conclusive. First, Dhouailly (1975) noted that, in combinations of chicken dorsal dermis and lizard epidermis from various parts of the body, abortive scale primordia formed which, in some cases were distributed in a pattern resembling the normal feather pattern, suggesting that the dermis determines the pattern. However, in this case, the evidence relies on a few observations where the pattern was not carefully analysed. Second, evidence which has been quoted in reviews (Sengel, 1976b) comes from experiments by Novel (1973) in which dermis and epidermis from chicken dorsal skin were combined in

90° relative rotation. However, since the normal feather pattern cannot be clearly distinguished from a 90° rotated pattern (Linsenmayer, 1972), these experiments cannot be interpreted in terms of the spatial pattern: they are, however, relevant to the control of the timing of morphogenesis and are discussed in this context below. Third, evidence that the dermis can determine the feather pattern comes from an experiment by Linsenmayer (1972) and applies to skin of the thigh where morphogenesis begins on the seventh day of development. Eight day-old epidermis which already contained placodes was combined with dermis taken from 6 day-old skin before morphogenesis had begun. Since primordia becomes more widely spaced as the skin grows, the placodes were spaced differently from the prospective spacing in the undeveloped dermis and thus the patterns expected of the two tissues could be distinguished. Primordia developed with the normal spacing, as expected if the dermis determines the pattern. This experiment is, however, open to criticism: the wide age difference between the two tissues, and the fact that one had already passed the stage when patterned morphogenesis is normally initiated, make interpretation of the result difficult. It would, perhaps, be better to combine tissues from distinctive, feather-forming regions: say anterior cervical dermis with lumbar epidermis, both taken before morphogenesis begins, since these would be expected to be closer in developmental stage. Nevertheless, if we accept that scale and feather patterns are determined by the same tissue type, we are on safer ground: Sullivan (1972) and Linsenmayer (1972) showed convincingly that tarsometatarsal dermis determines the scale pattern. For example, Sullivan showed that scale epidermis, combined with its own dermis in

90° rotation, formed scales in a distribution in accordance with the orientation of the dermis.

A single aspect of the final pattern is known to be determined by properties inherent in the epidermis close to the time of morphogenesis. Feather-buds elongate asymmetrically so that the feathers eventually lie back along the skin. Combinations of dermis and epidermis in 90° and 180° relative rotation have shown that the orientation of the feather-bud follows epidermal polarity (Novel, 1973).

These results deal only with the spatial aspects of the pattern. Which tissue determines the timing of morphogenesis? Bell, in 1964, showed that the onset of primordium morphogenesis is a tightly controlled, intrinsic property of the skin: when 4.75 day-old dorsal integument was grafted to the flank of a 3 day-old host, primordia formed at the time expected of the graft, 1.75 days before the host formed feather primordia. Linsenmayer (1972) showed that the time of eventual feather formation is already determined in 5 day-old thigh skin. The dermis also governs the temporal sequence of morphogenesis across the skin: when dermis and epidermis are separated and then recombined in 90° rotation before primordia have started to form, the position and orientation of the initial row and the spread of pattern across the skin are in accordance with the prospective pattern of the dermis (Linsenmayer, 1972). Dermal control of the timing of development applies, not only to the initial stages of morphogenesis, but also to much later events, for in 4 day-old chicken the mesoderm is the seat of properties which eventually govern the time of emergence of the second generation primary feathers (Cairns and Saunders, 1954; Dhouailly, 1970). The

literature on temporal aspects of feather primordium development - as is that on the temporal control of development in general - is fragmentary, and confined to incidental observations and isolated experiments.

2.4 Progressive Stages in Pattern-Determination

Early Events

Early events in the determination of the feather pattern have been studied mainly in the prospective tissues of the spinal pteryla, the dermal cells of which originate in the dermatomal parts of the somites (Straus and Rawles, 1953; Mauger, 1972a). It is known that, even before segmentation, the somitic mesoderm is organised in a manner which will eventually determine the region-specific size and spacing of primordia, the curvature of the chevrons, and - at least approximately - the lateral extent of the pteryla. The evidence for this important conclusion was obtained by Mauger (1972b). When somitic mesoderm, which was segmented or about to segment, was transposed from one level of the axis to another in 2 to 2½ day-old embryos, the pattern which eventually developed in the skin above the graft was characteristic of the pteryla at the level of origin of the graft. For example, transposition of posterior cervical somites to the thoraco-lumbar region caused an area of posterior cervical-type pattern to form in the thoraco-lumbar region, as judged in the 10 to 12 day-old embryo. It is interesting to note that at 2 days the somitic mesoderm is also regionalised with respect to the formation of the axial skeleton, (Keiny, Mauger, and Sengel, 1972). Early regionalisation is not confined to somitic mesoderm: the development of a pectoral pattern

in the lumbar region after replacement of several somites by somato-pleural mesoderm, indicates that the source tissue of the pectoral region is also regionalised at 2 to $2\frac{1}{2}$ days (Mauger, 1972a).

Precisely how this regional organisation arises is not known. However, tissue interactions around 2 days development appear to play a crucial part in the development of feather-forming properties in the somitic mesoderm. The dermatomes lie close to the neural tube, and the earliest factor known to influence specifically the development of feathers is an interaction between dermatomal cells and the neural tube at this time. The evidence suggests that brief 'contact' with living neural tube is required (Mauger, 1972c; Sengel, 1976b). In line with current evidence that tissue interactions in some other systems involve direct cell contact (Saxén et al, 1976; Nordling et al, 1977), Sengel (1976b) suggests that these interactions may be mediated by the 'contacts' which Trelstad, Hay, and Revel (1967) demonstrated to occur between somitic mesodermal cells and the basal lamina of the neural tube in the 2 day-old embryo. However, Trelstad et al also reported contacts between neural tube cells and the extracellular sheath of the notochord, so that tissue interactions involving cell contact in this area may be quite complex. Although these interactions may be necessary for feather development, the regional differences in the pattern do not seem to be imposed on the mesoderm by the neural tube, for transposition of the neural tube itself has no effect on the pattern (Mauger, 1972c).

Progressive, Stable Determination of the Mesoderm

All that is known about the pattern-determining organisation in the mesoderm is that during early development it shows considerable plasticity under experimental conditions, but loses this

property as development proceeds.

In the chicken, unsegmented mesoderm and the most recently formed somites are not irreversibly committed to form a particular tract of the spinal pteryla: after excision of 6 such somites, or of a length of unsegmented mesoderm, normal, or nearly normal patterns develop (Mauger, 1972a). Keiny et al (1972) reported similar findings in the development of the axial skeleton. This phenomenon appears to depend on the space vacated by excised somites being colonised by neighbouring cells (most likely from adjacent somites (Mauger, 1972a)): if a group of somites is unilaterally replaced by another tissue or by inanimate material, or if somites are irradiated and left in place, then a local apertium forms on the operated side (Mauger, 1972a; Mauger and Sengel, 1970). It seems, therefore, that cells of somites adjacent to the excision are capable of regulation[1] with respect of their regional, pattern-forming properties. This regulative capacity is gradually lost: excision of somites at progressively later stages results in pattern aberrations of increasing severity (Mauger, 1972a).

The organisation responsible for the polarity of somitic mesoderm in the different embryonic axes also becomes progressively fixed. Mauger (1972b) investigated the establishment of the axes by

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[1] Regulation. A certain degree of organisation (a field (for reviews, see Waddington (1966, 1968)) has arisen in the mesoderm which integrates the committment of cells to specific developmental pathways, and thus leads to the development of a particular feather pattern. Following a disturbance, the mesoderm may become reorganised in a way which tends to preserve the unity of its development. Such reorganisation is termed regulation. Though field phenomena are common in development, the nature of the organisation which underlies them is not known.

excising a length of somites (or unsegmented mesoderm), then placing it in a host chicken so that only one axis was inverted. The medio-lateral axis is the first to become established, around the time of segmentation. Inversion of the dorsoventral axis at this time, however, revealed a striking capacity of the ventral (sclerotomal) somitic tissue for regulation. Histological evidence showed that cells (presumably from the now dorsal sclerotome) rapidly contributed to the subectodermal mesenchyme. About half these specimens developed normally, and Mauger concluded that inversion of the dorsoventral axis has no effect on feather formation. According to Sengel (1976b) plumage defects gradually appear as older somites are rotated, indicating a loss of regulative capacity. Finally, inversion of the anterio-posterior axis had no effect on the feather pattern at the stages studied, excepting the consequences of the necessary transposition of somitic material along the axis.

The Movement of Mesodermal Cells to the Integument

The early stages in the development of the presumptive dermis have been described by Sengel (1971). In the $2\frac{1}{2}$ day-old embryo, the integument comprises a layer of ectoderm beneath which is a space, free of cells, and occupied by a fibrous matrix. From about 3 to 5 days of development, cells of the source mesoderm of both pterylae and apertia migrate to the integument to colonise this space and gradually build up a loose subectodermal mesenchyme of which the cells immediately below the ectoderm will later form the dermis. Little is known of the precise nature or route of these cell movements. However, the distances involved are not great: the integument is typically in the order of 30 to 300 µm from the source

mesoderm. By 5.5 days of incubation (Mauger, 1972a), cells from the disintegrating dermatomes (Trelstad et al, 1967; Hay, 1968) have colonised the entire width of the spinal pteryla.

To follow the development of the factors determining the regional qualities of the feather pattern, we must therefore turn to the organisation of the subectodermal mesenchyme.

The Regional Organisation of the Subectodermal Mesenchyme

In no case is it known how large or homogeneous are either the original mesodermal regions, or the populations of cells in the subectodermal mesenchyme derived from them. However, some information is available on the sharpness of boundaries between cell populations and on the general distribution of cells of different origin in the subectodermal mesenchyme.

The most precise information relates to the lateral margin of the spinal pteryla in the flank. By substituting for a group of host somites, somites labelled with tritiated thymidine or quail somites (the cells of which possess a distinct nuclear marker), Mauger (1972a) showed that cells from the somitic mesoderm form an abrupt boundary with latero-ventral mesenchyme of somatopleural origin about half-way down the flank. Mauger's photographs suggest that, in the 5.5 day-old embryo, this boundary is in the order of 50 μm wide. The boundary would thus be sharp enough to define the transition between pteryla and apertium to within less than the width of a single row of primordia. Indeed, the less precise data of Mauger and Sengel (1970), based on carbon-marking experiments, indicates that this cell population boundary does correspond approximately to the lateral margin of the spinal pteryla in the flank region. In this case, therefore, it seems that the margin of the

pteryla could, in principle, be determined directly by the distribution of distinct populations of subectodermal cells.

The situation is more complex, however, in the cervical region of the spinal pteryla, for here the approximate boundary of somitic cell migration lies well within the apertium (Mauger and Sengel, 1970). This low-resolution analysis shows that somitic origin per se is not a sufficient condition for the participation of cells in the spinal pteryla. However, without more precise data on the origin of the cells, no conclusions can be drawn regarding the relation of the pteryla boundary to the regionalisation of the subectodermal mesenchyme in this region.

The distribution of cells of different origin within the spinal pteryla was also examined by Mauger and Sengel (1970). Carbon-marking showed that each group of about three marked somites contributes to a well-defined part of the pteryla. Groups of 3 somites in different regions contribute to parts of the pteryla of different length, varying from the equivalent of one to nine primordia in the initial row. The situation is further complicated by caudal displacement of cells in relation to the axial elements of the same origin. However, the marking and mapping methods employed were of insufficient resolution to define the precise boundaries of differently determined cell populations. Moreover, even the general distribution of cell populations at the important stages when the subectodermal mesenchyme is being formed and immediately before primordia emerge cannot be assessed directly because the results were analysed only in the 11 day-old embryo.

One further difficulty in interpreting these results is that they are apparently contradicted by a second series of experiments in which Mauger and Sengel used X-rays to cause local lesions

along the embryonic axis over successive lengths of about 3 somites. Well-defined strips of bare skin developed, not caudally, but at the level of treatment; the length of each strip corresponded to the length irradiated. In itself, this experiment is difficult to interpret, for all the axial and overlying tissues in each region were irradiated and it is not clear what was the primary effect of the treatment. However, it is likely, in any case, that the somitic cells were destroyed or damaged and it is therefore difficult to explain why the bare strips formed at the irradiated level and did not extend caudally. In the face of these difficulties caution must be exercised in interpreting the results of either set of experiments in terms of the necessary contribution of cells from particular somites to the formation of the feather pattern at specific levels of the spinal pteryla.

In conclusion, the only available high-resolution data suggests that the boundaries of the pteryla in the flank region could be directly defined by the precise distribution of regionally determined cell populations in the subectodermal mesenchyme, data from other cell marking experiments are not of sufficient resolution to test this possibility more generally.

Progressive, Stable Determination of the Integumental Pattern

It is clear from the following evidence that certain aspects of the feather pattern are progressively determined precisely in space as the integument is being built up, although the relationship of this determination to the distribution of subectodermal cell populations has not been specifically examined.

The detailed outline of the feathered area and regional characteristics of feather structure are progressively determined in

the humeral area during a period about three days before primordia begin to form. This was shown in an important series of experiments by Saunders and Gasseling (1957). Their approach was to excise a thin rectangle of integument from the 3 to $4\frac{1}{2}$ day-old wing bud, rotate it through 180^{o}, and replace it in the wing bud. In this way, part of the prospective humeral pteryla - including part of the region where we would expect the prospective site of the initial row (Holmes, 1935) - came to lie in the distal-adjacent apertium, and vice versa. Normal patterns developed in some embryos operated at each stage - particularly where the area rotated was small, and after operations at Stages 17 to 21 - suggesting that the integument is partially organised, but capable of some regulation, at this stage. Typically, however, disturbances in the pattern resulted which suggest that the precise limits of the feather-forming area become progressively determined up to Stage 20 to 24. Operations at Stages 17 to 19 most frequently produced a small bare patch, equivalent to part of one or of a few rows, at the distal margin of the humeral pteryla, or caused a general reduction in the size of the pteryla; later operations most often caused a similar abnormality in the pteryla and, in addition, produced a small tuft of feathers in the adjacent apertium. The feathers in this tuft corresponded approximately in number to those missing from the pteryla, suggesting that the extent of the pteryla is determined precisely at these stages. Furthermore, the structural characteristics of the adult feathers in the tuft resembled, in detail, those normally found in the distal part of the humeral pteryla. The results also suggest that the temporal control of later steps in feather development is already inherent in the organisation of the integument at this stage. Juvenile feathers

in the tuft emerged simultaneously with those in the pteryla, even though the two groups were typically separated by a strip of bare skin. This early organisation affecting the timing of morphogenesis may also apply to the formation of the rows of primordia themselves. In the embryonic specimens examined the distribution of the sizes of primordia in the tuft was equivalent to that of the humeral pteryla inverted proximo-distally: the size distribution of primordia generally corresponds to the sequence of morphogenesis across the pteryla (Holmes, 1935; Fraps and Juhn, 1936; Linsenmayer 1972). Finally, the results suggest that the anterio-posterior polarity of this part of the integument is fixed during the period between Stages 22 and 24. In tufts formed as a result of operation before this time, the feathers pointed posteriorly, but in some specimens operated after Stage 22 a few feathers pointed anteriorly, in conformity with the orientation of the rotated piece.

The evidence reviewed in Section 2.3 suggests that all of these characteristics of the pattern are determined by the prospective dermis, except the orientation of feathers which is possibly defined by ectodermal polarity. However, the results do not indicate specifically that the whole area is organised with respect to the eventual feather pattern at this atage. Alternatively, it may be that only the position and characteristics of the regional boundary, or of the initial row, are defined and from these the pattern emerges later in development.

The evidence reviewed thus far has established that the outline of the tracts of feather pattern and the regional differences between them are determined by properties established early in development, although several important questions remain unanswered;

in particular, what properties distinguish the mesoderm in different regions, and how does the eventual size and shape of feather tracts relate to the pattern of mesodermal colonisation of the subectodermal space? It is not, however, known when and how the positions of individual primordia are determined, though the view is generally held that these are determined close to the time of morphogenesis (Linsenmayer, 1972; Stuart, Garber, and Moscona, 1972; Novel, 1973; Sengel, 1976b). The evidence for this is examined in the following section.

Are the Positions of Primordia Determined Before Morphogenesis Begins?

The determination of feather positions in the initial row will be considered first.

In their study of the structure and development of the spinal pteryla, Mauger and Sengel (1970) considered the possibility that the number and position of primordia in the initial row are determined precisely by the early regionalisation of the integument. They compared the number of initial primordia with the number of underlying vertebrae (which corresponds to the number of somites) in the 11 day-old embryo. Though each of the 18 initial primordia in the lumbo-sacro-caudal region corresponds precisely to one underlying vertebra, a 1:1 relation does not hold over the entire pteryla. From this evidence, Mauger and Sengel concluded that the integument is not metamerised. The absence of a universal 1:1 relation does not, however, exclude the possibility that the number and position of primordia in each region of the initial row are closely controlled by the regional organisation of the integument. Mauger and Sengel suggest that the 1:1 relation in the posterior regions is fortuitous and, certainly, it poses problems. The chief

problem arises from the finding that the integument moves caudally in relation to the axial elements from which it is derived, and spreads to different extents in different parts of the presumptive pteryla. Thus, constraints arising from the original segmentation of the source mesderm would not simply account for the close morphological relation between initial primordia and the underlying vertebrae in the 11 day-old embryo. However, as is shown on page 21, there is conflicting evidence on this point. The anterior cervical region of the pteryla, which shows the highest number of initial primordia per vertebra, shows the greatest axial growth over the total period studied, and this led Mauger and Sengel to conclude that the number of initial feathers in each region depends on the axial growth of the region between the stage when somites individualise and 11 to 12 days of incubation. Their data for other regions do not, however, fit this conclusion in a simple way (Mauger and Sengel, 1970: Table 2). Moreover, skin growth between 7 and 12 days of incubation is irrelevant to the number of initial primordia, almost all of which have formed before this time. Until more is known about the precise distributions of cells in the integument and their patterns of growth up to the time when the positions of primordia are established, this interpretation is in doubt. It is therefore best to leave open the question of whether the 1:1 correlation in the posterior tracts is fortuitous or reflects a more general, tightly controlled relation between each body segment and a certain number of initial, medial primordia.

We may now consider when the positions of the remaining primordia in the pteryla are determined. The best evidence regarding this comes from experiments on the development of the thigh pteryla (Linsenmayer, 1972). Equal-sized pieces of integument,

excised from young (4.5 to 5 day) and older (6 to 6.5 day) thigh pterylae before the onset of primordium morphogenesis, were cultured on the CAM. In vivo, the prospective pteryla grows in area by a factor of about $3\frac{1}{3}$ in the period between 4.5 to 5 days and 6 to 6.5 days development. During the same length of time explants grow far less in area. Linsenmayer argued that if the feather pattern is latent in the integument around 5 days of incubation then $3\frac{1}{3}$ times the number of primordia will form in the younger, than in the older, explants.

In fact, the measured spacing between primordia (the length of the first row to form divided by the number (2 to 4) of primordia in it) was the same in both young and older explants. The total number of primordia formed in young explants was far short of $3\frac{1}{3}$ times the number formed over approximately the same area in older explants. Linsenmayer interpreted these results to indicate that the pattern is not determined several days before primordia form. Before primordia began to form, some of the older explants grew to twice the size of others, yet primordia eventually formed with the same spacing. This led Linsenmayer to suggest that the positions of primordia may not be established even by 6 to 6.5 days of incubation. However, this latter evidence is weak for the result must be interpreted with caution: growth of an explant on the CAM may be due mainly to an unco-ordinated extension of the marginal tissues, rather than to an increase in the feather-forming area such as occurs in intact skin.

Clearly, the results of these experiments indicate that the positions of primordia can be influenced by experimental manipulation about 2 days before morphogenesis begins. No point-by-point determination of the pattern is already present which is

irreversibly committed to complete expression. It might be argued, however, that this experiment imposes severe spatial restrictions on the expression of any predetermined pattern which might be present. Indeed, the morphogenetic process may be biased against producing one-third normal spacing. This is supported by the evidence that primordium spacing is already determined in the regional properties of the source mesoderm (Mauger, 1972b). The possibility therefore remains open that a predetermined pattern is present but undergoes regulation under these conditions, or that only a proportion of pre-established sites develop.

A re-examination of Linsenmayer's results suggests that the integument may not be entirely unpatterned two days before morphogenesis. If, at the time of explantation, the pieces of integument were entirely equivalent as regards constraints on the future positions of primordia then young and older explants would be expected to form the same number of primordia per unit area. However, Linsenmayer's results do indicate that, while the feathered areas are not significantly different in size (and may therefore be taken as unit area), a significantly greater number of feather primordia were formed per unit area in younger, than in older, explants (Table 1.1). This implies that there may have been a difference in the size and spacing of primordia in the two types of explant. That this difference is not shown by the measured spacing of primordia in the first row may be due to inaccuracies inherent in these measurements: the possibility of such inaccuracy is discussed by Linsenmayer.

Thus, Linsenmayer's results suggest that a certain degree of organisation may be present by $4\frac{1}{2}$ to 5 days which is sufficient to push morphogenesis towards forming a greater feather density when

TABLE 1.1 Analysis of Results Obtained by Linsenmayer (1972).

Feather primordia formed in equal-sized pieces of young and older thigh skin cultured on the CAM.

Explants	Mean Values	
	Area over which primordia formed (mm^2)	Number of primordia
9 young (4.5 - 5.0d)	3.37 ± 0.97	34.4 ± 7.66
13 older (6.0 - 6.5d)	3.04 ± 1.18	24.8 ± 3.95

The values for different explants may be compared using the t-test modified for two small populations of unknown, equal variance (Bailey, 1959; page 47). Linsenmayer does not give the meaning of figures after the ± sign. However, if these figures are assumed to be one standard deviation, the difference between the areas with feathers is not significant at the 5% level (estimate of $t = 0.692$ with 20 degrees of freedom (DF)), while the difference in the number of primordia formed in young and older explants is significant at the 1% level (estimate of $t = 3.86$ with 20 DF).

a piece of the pteryla develops without the normal degree of growth. Of course, the nature of this organisation, and whether, or not, it leads to preferred positions for the development of individual primordia remains to be shown.

The only other piece of evidence relating to the time at which the positions of primordia are determined comes from studies by Sengel and Novel (1970) and Novel (1973), but the results are difficult to interpret. When skin in which several rows of primordia had already formed was placed in certain culture media and subjected to experimental manipulation (for example, if the dermis and epidermis were separated, or the initial row destroyed), existing primordia seemed to disappear and an apparently new pattern formed. These results were interpreted to indicate that the pattern is established only as morphogenesis occurs across the pteryla. However, this evidence is weakened by the lack of a clear histological demonstration that primordia do disappear completely, and by the difficulty, recognised by Novel, of distinguishing a new spatial pattern from the re-emergence of the previous one.

In conclusion, though the positions of primordia throughout the thigh pteryla are not irreversibly established before two days prior to morphogenesis, the current view (Linsenmayer, 1972; Novel, 1973; Sengel, 1976b) that their positions are entirely determined close to the time of morphogenesis rests on insubstantial evidence.

2.5 Contemporary Models of Feather Pattern Formation

An explanation of the mechanism of feather pattern formation should account for three aspects of the phenomenon: first, the periodic nature of the pattern; second, the sequence of pattern expression; and finally, the positions of individual primordia. In

addition, the essential features of the model would be expected to account for the patterns of other skin appendages.

Three models have been published to explain the development of the feather pattern (Ede, 1972; Stuart, Garber, and Moscona, 1972; Novel, 1973). There has been no critical review of these and no attempt to test them experimentally. However, the assumptions they rest on and the predictions they make raise important questions about the constraints which govern the time and position of primordium morphogenesis.

Template Propagation of a Regular Diamond Pattern

The nature of these three models rests on two general assumptions. First, that the position of each primordium is determined immediately in advance of morphogenesis in hitherto unpatterned skin. This assumption stands in contrast to the interpretation of pattern formation in several other systems where the evidence suggests that the site at which organs will form depends crucially on organisation established in the tissue some time before morphogenesis begins (Stern, 1954; Wolpert, 1971, 1977). Second, the models assume that the repeated, neighbouring units of the pattern are equivalent. This need not be so, and would not be so were their determination derived from a mechanism based on positional information (Wolpert, 1969; Lewis and Wolpert, 1976).

The particular postulates of the models derive from an examination of the way feather primordia emerge. Since morphogenesis occurs sequentially across the pteryla, it has been suggested that the pattern is propagated row-by-row through the dermis (Ede, 1972; Stuart et al, 1972; Novel, 1973). This view embodies two distinct ideas: first, that some stimulus or permissive condition to form primordia spreads through the dermis immediately in advance of

primordium formation; and second, that the regularity of the pattern itself is propagated by a template mechanism in which primordia in one row determine the positions of primordia in the next. The activation to form primordia and the determination of their position within a row need not be governed by the same mechanism.

Of the three published models, the first two which will be considered - those of Ede and Novel - are based on one further consideration; that the feather pattern approximates to a close-packed, regular array. Novel (1973), for example, likens the pattern to an hexagonal array, an arrangement which, she notes, accommodates a maximal number of elements in a given area. The models therefore set out to explain the pattern using space-filling constraints. The geometric regularity of this approach, and the assumption that primordia are equivalent, implies that each new primordium is equidistant from two neighbours in the preceeding row.

The Diffusion-Reaction Model (Ede, 1972)

This approach invokes the action of concentration - patterns of diffusible substances on the morphogenetic and biochemical activity of cells. It has its origins in the 1940s and 1950s when chemical gradients were first thought likely to determine patterns in the embryo (Child, 1941). The first major step was made by Turing (1952) who showed how an initially homogeneous system of two or more diffusible and cross-reacting chemicals ('morphogens') could develop periodic heterogeneity after small, random disturbances. He suggested that such a chemical distribution could form the basis of patterned morphogenesis. About this time, Wigglesworth (1953) suggested a model for the regular spacing of bristles in the bug, Rhodnius: that each bristle site, as it formed, utilised precursors from the surrounding tissue and thus, by competition, inhibited

bristle formation within a certain area around it. Claxton (1964) used a similar scheme to account for the pattern of follicles in sheep skin which is of the type exhibited by pelage hairs described in Section 2.2. Though he did not examine the chemical nature of the process, Claxton showed that if one takes account of skin growth and the possible variation in the radius of effective inhibition it is possible to simulate the patterning process and arrive at realistic values for the range and variance of nearest neighbour distances within the pattern. He also applied this approach to the patterns of hairs in camels and mice and of microchaete bristles in Drosophila. All these patterns, however, are irregular (see page 8).

Ede proposed a model for feather pattern formation which built on Claxton's approach. According to this model, a zone of inhibition is generated radially round each forming primordium, by the production of a diffusible inhibitor, or by competition for a necessary, diffusible substance. New primordia would only form outside a circular threshold contour of inhibition round existing sites. If inhibition decreases steeply across this threshold contour, and some mechanism exists to guarantee primordium formation outside the zone of inhibition these conditions would give the pattern a well-defined periodicity. To account for the regular array of primordia, Ede assumed the temporal sequence of morphogenesis by suggesting that competence to form primordia is confined to a narrow, but ever-widening band of dermis lateral to already-formed primordia. This introduces a packing constraint: new primordia would form as soon as sufficient competent dermis became available. Since the first point to become competent and available would be at the intersection of adjacent threshold contours, new primordia would form equidistant from two neighbours in the

preceeding row.

Ede suggested that the spread of competent dermis depends on lateral diffusion of an activator substance from the midline. However, this would result in an exponential decrease in the rate of initiation of new rows with distance from the midline (applying the general arguments of Crick (1970)). In fact, initiation appears to be approximately constant, at least over the first few rows (Hamburger and Hamilton, 1951). This difficulty could be simply overcome by suggesting that the key substance is produced by successive bands of activated dermis. Since the distances between successive rows are small (about 200 to 300 μm), the model is plausible in terms of timing (Crick, 1970). Ede also suggested that a substance diffuses backwards from the head region initiating primordia first anteriorly, then posteriorly. However, the reverse sequence occurs over part of the midline in the spinal pteryla (see page 5). But this seems an unnecessary complication. Constraints which are taken for granted by the model are required to specify the location and orientation of the initial row and the site of the first primordium, which consistently forms in the same region (see page 5). If the site of only the first primordium is determined initially, then formation of the remaining primordia in the initial row may be governed by anterio-posterior diffusion, from this site, of the same substance as later governs the lateral development of primordia.

Perhaps the most important merit of this approach is its potential application to a wide range of patterns. Irregular skin appendage patterns could be accounted for by Claxton's scheme and since most, if not all, the regular skin appendage patterns emerge in a strict temporal sequence, these could apparently be explained by

Ede's scheme. Moreover, there is some evidence that competitive or inhibitory mechanisms may operate in other systems where periodic patterns are found; for example, in the formation of budding in hydroids (Berking, 1977), and in plant systems where the positions of leaf primordia during phyllotaxis may involve the action of diffusible inhibitors (Wardlaw, 1968 ; Mitchison, 1977; Richter and Schranner, 1978), while the periodic spacing of heterocysts in the filamentous alga, Anabena, may involve competitive chemical interactions (Wilcox, Mitchison, and Smith, 1973a). Moreover, Gierer and Meinhardt (1972) have shown the possibility of applying essentially similar, but more complex, schemes to pattern-forming systems in which a latent pattern appears to be present before morphogenesis begins and is capable of regulation after changes in tissue area.

The principal difficulty with this model is that, while it shows how the pattern <u>can</u> be generated, it is very difficult to test, experimentally, whether or not this mechanism <u>is</u> responsible for the feather pattern. Many schemes using slightly different ingredients can, in theory, generate a similar pattern. Moreover, considerable problems arise in obtaining positive evidence of the presence, distribution, and action of the very small amounts of hypothetical morphogens involved.

<u>Steric Inhibition (Novel, 1973; Sengel, 1976b)</u>

This second model proposes that existing primordia influence the position of incipient sites by steric, rather than chemical inhibition. The model is based on the observation that the primordia in a newly-formed row are very close to their neighbours in the preceeding row (Sengel, 1971). To explain the periodic nature of the pattern, it is assumed that "feather-forming dermal cells

possess the intrinsic, genetically determined property to group themselves into lens-shaped condensations of given diameter and cell density" (Sengel, 1976b; page 247). The way in which this scheme accounts for the regular feather array closely resembles the model of Ede. The key assumption is again that competence is attained in a strict temporal sequence across the pteryla and is confined at any time to a narrow band of dermis, so that new primordia form as close as is physically possible to two neighbours in the preceeding row. However, in the spread of competence this model favours relatively direct cellular interactions: 'morphogenetic activity' is "passed in a wave-like fashion from cell to cell from the middorsal line toward the lateral edges of the spinal feather field" (Sengel, 1976b; page 251). Finally, it is necessary, as Sengel has pointed out, to postulate that the first primordium is located by a different (and unknown) mechanism.

At first sight, this model might appear to offer a more direct explanation of pattern-formation than the diffusion-reaction model. Indeed, this was the main reason for its proposal: Novel considered that the steric model can more reasonably explain regional differences in primordium size than can a diffusion-reaction scheme. The model is not, however, explicit as regards how the proposed physical constraints between sites actually operate. How are dermal cells integrated in such a way that the completion of a critical area of competent dermis triggers primordium formation? Is the position of a new primordium initially defined in terms of its boundary or its centre? What kind of physical interactions between the dermal cells are involved? These considerations make it clear that the situation is no simpler than the one envisaged in the reaction-diffusion model.

They also illustrate a weakness of this model in that it is too descriptive and the problems and possibilities inherent in it are not explored.

Close apposition of primordia is not a property shared by all skin appendage patterns; in exceptions, primordia do not form closer together than a certain distance which is characteristic of the pattern; mammalian hair patterns (Claxton, 1964) may be cited as an example. Moreover, in some patterns where primordia do form close together - for example, Reptilian and Avian scale patterns - there is no evidence from histological descriptions that dermal structures are formed which could be expected to interact sterically between primordia (Maderson, 1965; Dhouailly, 1975; Sawyer, 1972a). Sengel (1976b) considers that a different mechanism of pattern determination may operate in other systems. However, the evidence that the pattern-forming mechanism for all skin appendages is essentially similar presents a serious difficulty for this model, although it cannot be entirely ruled out that steric interaction is derived from structures in these other patterns which are not obvious histologically.

The Patterning Rôle of Dermal Arrays (Stuart et al, 1972)

The third model is based on one further observation: histological evidence indicates that, between formed primordia, elongated dermal cells are mutually aligned to form 'arrays' (Stuart and Moscona, 1967; Stuart et al, 1972). These arrays intersect at condensations. According to Stuart et al, (1972), extracellular fibres are also aligned within these arrays and this arrangement of fibres and cells becomes progressively organised in the dermis lateral to already forming condensations, so that diagono-lateral

arrays intersect at the sites of new primordia. These observations led Stuart et al to propose the following model.

The sequence of morphogenesis is accounted for by the progressive organisation of the dermis into arrays which extend diagono-laterally from formed condensations to form a lattice of oriented cells and fibres in hitherto unpatterned skin. The intersections in this lattice define the sites of new primordia. Cells move along the fibres in these arrays to intersections where their movement is restricted and there they form clusters which enlarge into condensations. Stuart et al do not suggest how the periodic structure of the initial row forms, though they do point out that, in the spinal pteryla, its development follows the formation of an array along the midline.

This model is explicit in its account of the structures involved, and it thus has the merit of being open to critical experimental test. Moreover, it has the interesting property of accounting for the observed histological structure of developing dermis in a way which links the formation of the feather pattern with the morphogenesis of individual primordia.

However, the model suffers serious difficulties. First, there is conflicting evidence on whether the arrays actually extend lateral to the most recently formed rows of primordia (Wessells and Evans, 1968; Ede, Hinchliffe, and Mees, 1971) and it is not clear to what extent cells move along fibres in these arrays. Second, the particular evidence which Stuart et al present in support of their model is open, on each point, to reasonable alternative explanations. These two difficulties are discussed in detail in Section 3 , since they are best dealt with in the context of dermal morphogenesis. Third, the model focusses on features which appear

to be unique to the feather system: arrays have not been reported in the superficial dermis in histological accounts of the development of other skin appendages whether these do, or do not, form condensations for example, in mouse vibrissae (Wessells and Roessner, 1965) or in chicken or Reptilian scales (Sawyer, 1972a; Maderson, 1965; Dhouailly, 1975). There is thus no reason to suppose that this mechanism operates in the determination of other appendage patterns.

The following subsections examine evidence relating to the common assumptions of these models.

Evidence Concerning the Importance of the Initial Row

The models assume that the development of each successive row of primordia depends on the passage of a state of activation or competence across the pteryla immediately in advance of morphogenesis.

Evidence obtained by Sengel's group has been interpreted to support this assumption. This focusses attention of the importance of the initial row. A range of different experimental treatments -- X-irradiation or excision of the neural tube in discrete axial regions of the 2 day-old embryo (Sengel and Keiny, 1963; Mauger and Sengel, 1970; Mauger, 1972a), or hydrocortisone injection of 5 to 6 day-old embryos (Sengel and Züst, 1968; Züst, 1971) -- causes varied, and more or less severe, plumage dificiencies; however, in all cases, wherever medial rows of the spinal pterylae are absent, lateral rows also fail to develop. (The effect of X-irradiation is to destroy the source cells of the pteryla, though it would also affect other tissues since the treatment was not delivered specifically to somites; the crucial importance of the neural tube has been dealt with in Section 2.4; hydrocortisone acts

on a variety of systems, including cell proliferation in the dermis and epidermis and the organisation of collagenous and polysaccharide structures (Züst, 1971; Roberts, Karnofsky, and Frankel, 1951; Dziewiatkowski, 1964; Gould and Manner, 1967), but its primary effect on the feather system is not known.) Sengel (1976b) interprets these results to indicate that the formation of the initial row is the origin of a 'wave of morphogenetic activity' which spreads through the pteryla, the development of each successive row activating the formation of the next. However, the evidence must be interpreted with caution for none of these treatments has been shown to, or is likely to, specifically affect the initial row.

There is, however, clear evidence that lateral rows can form without continuity with the area of skin containing the initial row. Evidence for this was obtained by Linsenmayer (1972). Before the onset of morphogenesis, a piece of thigh skin was severed from the region where the initial row normally forms, and was cultured in isolation on the CAM. A normal diamond pattern developed in this piece of skin; the first row formed at the margin which had been nearest the presumptive site of the initial row in the intact pteryla. As Linsenmayer has pointed out, these results do not distinguish between two alternative possibilities. The first of these is that the first row in the severed piece forms by the same mechanism as would operate in an equivalent region in uncut skin. This would imply that the activation to form primordia is not propagated through the skin close to the time of morphogenesis. The second possibility is that the first row may be a new 'initial row' with special pattern-initiating properties, formed as a regulative response to cutting by a process which does not normally occur in an equivalent part of intact skin. For example, a new high

region in a gradient of polarity potential (Wolpert, 1969) may be set up at the cut edge which would initiate the propagation of an activated state beginning at this margin. Linsenmayer has suggested that these two possibilities might be distinguished by comparing the time at which the first row of primordia form in a severed piece of skin with the time at which primordia form in an equivalent region of the intact pteryla: for example, if the time of morphogenesis was changed by cutting the skin, this would favour the latter hypothesis. This type of experiment is clearly a potentially important way of exploring the kind of organisation which is responsible for the sequential timing of morphogenesis and will be considered further in Chapter 4.

Template Propagation of Pattern Irregularities

The models also lay emphasis on the template-like propagation of constraints on the position of new primordia as morphogenesis occurs across the skin: observations on the naturally-occurring pattern irregularities bear on this point.

By far the most common naturally occurring irregularity of the feather pattern is the addition or depletion of a continuous part of a single chevron, beginning at any distance from the initial row and extending to the edge of the pteryla. These irregularities have been noted specifically by Linsenmayer (1972) and are also evident in many published photographs of the feather pattern (see for example, Sengel (1976b)). The continuation of every irregularity through successive rows to the edge of the pteryla suggests that the pattern may be propagated by local interactions between primordium sites in the same direction as the sequence of morphogenesis proceeds. However, it is important to note that these results do not otherwise

link pattern formation with morphogenesis or indicate when propagation of the pattern occurs. The addition or depletion of individual primordia is consistent in a simple way with the space-filling models of Ede and Novel, but is more difficult to account for by the model of Stuart et al.

Regional Variations on the Regular Diamond Pattern

Although these models set out to account for a regular diamond pattern, published photographs and descriptions (Mauger and Sengel, 1970; Linsenmayer, 1972; Sengel, 1976b) clearly indicate that the pattern in the 10 day-old embryo shows regional variations on this simplified form (Section 2.1). In particular, the curvature of the chevrons raises an important point. Although chevron curves may arise secondarily by differential growth after a regular diamond array of primordia has formed (Sengel, 1976b), whether or not this does occur has never been firmly established. The only detailed descriptions of large areas of the pattern refer to embryos examined between two and three days after the last primordia in the pteryla have formed. Clearly, examination of the newly-formed pattern is required, and this will be described in Chapter 4.

Problems Regarding the Time of Dermal Morphogenesis

One final point is of particular importance to the models of Novel and Stuart et al. Like the model of Stuart et al, Novel's model implies that physical interactions between dermal structures are involved in establishing new primordium sites, for it is the dermis which determines the pattern. However, there is good evidence that the epidermal placode develops before the dermal condensation (Sengel and Rusaouën, 1969). For the model of Stuart et al, this clearly would require that arrays of cells or oriented fibres intersect at the primordium site before epidermal

morphogenesis begins. For Novel's model, it would require that the most recently established primordium sites showing epidermal morphogenesis are associated with dermal structures capable of exerting steric constraints on the position of the next sites in the pattern.

It is therefore of crucial importance to examine the time of dermal morphogenesis in relation to the time at which localised changes in the epidermis signal that the positions of primordia have been established.

2.6 Interim Conclusions

It is clear that the most immediate question which needs to be answered in relation to the first of the problems posed in Section 1 is whether the sequence of morphogenesis and the position of individual primordia are already established throughout the pteryla before the first primordia form or are determined immediately in advance of the formation of successive rows. In particular, it is necessary to examine critically the rôle of dermal morphogenesis in determining the time and position at which new primordia are initiated.

Evidence relating to these questions will be presented in Chapter 4, but it is first necessary to review the evidence concerning the initiation and development of individual primordia.

3. THE DEVELOPMENT OF INDIVIDUAL FEATHER PRIMORDIA

This section deals with the initiation and development of individual primordia, with particular reference to the process of dermal morphogenesis. As background, the processes leading up to

primordium development are described in the first part of the section, while the evidence regarding the importance of tissue interactions in primordium formation are described in the second part. The third part of the section leads on from the arguments presented in Section 2 by examining the evidence regarding the time of dermal morphogenesis in relation to epidermal development, and the possible rôle of dermal cell patterns in determining the sites of new primordia. Finally, the evidence on the development of primordia at established sites, is reviewed.

3.1 Processes Leading up to Primordium Formation

Histogenesis of the Skin: Descriptive Evidence

The morphological development of the integument follows a similar path in both presumptive pterylae and apertia until after the fifth day of incubation (Sengel, 1971). As early as 2 days of development, a basement membrane-like structure is present below the two-layered ectoderm (Sengel and Rusaouën, 1969). After the beginning of the third day the matrix-filled space below this membrane is progressively colonised by secondary mesenchymal cells migrating from the source mesoderm (see Section 2.4). The morphology of these cells as they build up a loose mesenchyme has been described by Sengel and Rusaouën (1969), Meanwhile, the ectoderm continues to differentiate (Sengel, 1971), so that, by the fifth day, the upper layer forms a continuous, squamous periderm, while cells of the lower layer remain stellate and separated by conspicuous spaces. The periderm has a temporary, presumably partly protective, function and is sloughed off during late embryonic development (McLoughlin, 1961).

Over the period between 5 and 9 days, the integument in prospective feather-forming areas undergoes well-defined transitions which occur only later in apertia: the immediately subectodermal mesenchyme develops into dermis, and the undifferentiated, lower layer of the ectoderm, into epidermis (Sengel, 1971). Since these tissues form the starting point for feather development, it is worth describing their main features.

The development of the epidermis begins with the closure of intercellular gaps so that a continuous epithelium is formed (Sengel, 1971). The cells gradually elongate to a somewhat columnar shape about 9 μm high (Wessells, 1965). The ultrastructure of the basal epithelium, which is unremarkable, has been described by Kischer (1968) and Sengel and Rusaouën (1969). The periderm maintains the distinctive morphology of a pavement epithelium, being a single layer of tightly apposed, flat cells whose free surface bristles with short microvilli (Kischer, 1968; Sengel and Mauger, 1976).

The quite rapid transition from loose mesenchyme to true dermis involves several changes in tissue structure, the most obvious of which is an increase in the overall density of the tissue as judged in histological preparations: hence the tissue has been called dense dermis (Wessells, 1965). Two factors contribute to this dense appearance. First, the cell population density increases (Wessells estimated an increase to 1.3 - times the density of loose mesenchyme): the cause of this increase has not been investigated though it occurs even as the skin grows in area and must therefore involve an increase in the mean net rate of cell proliferation. Second, extracellular materials are produced, apparently uniformly through the tissue: this includes the synthesis of acid

mucopolysaccharides of unknown function or structure (as assayed autoradiographically and histochemically (Sengel, Bescol-liversac, and Guillam, 1962). However, in contrast to later stages in development, collagen is not present in sufficient quantity to be detectable histologically (Ede, Hinchliffe, and Mees, 1971), though collagen fibres have been observed in the transmission electron microscope (TEM) at this stage (Kischer, 1968).

One further morphological aspect of the changing structure of mesenchymal matrix is clear from electron microscopy studies. Bundles of extracellular fibres extend perpendicularly from the tips of sharp epidermal intrusions (spurs) into the dermis (Wessells, 1965). At this stage the spurs appear to be distributed randomly. Kallman, Evans, and Wessells (1967) showed that the bundles, which are in the order of 1 μm thick, are composed of thinner, parallel filaments, but the composition of these is not known. They do not, however, appear to be of mature collagen, for they lack its characteristic 64 nm periodicity. This kind of structure occurs widely in other animals: similar, but thinner, bundles of filaments are present in a variety of skin tissues in Amphibia and in rats and humans (reviewed by Palade and Farquhar, 1965, and by Kallman et al, 1967). However, it is not ubiquitous in the chicken skin: filament bundles were not detected beneath the smooth dermal-epidermal interface of tarsometatarsal skin (Sawyer, 1972a).

Little is known of how these structures form, though their development is not dependent on normal dermal morphogenesis and may be under epithelial control for if dermal development is prevented by destroying the somites both filaments and spurs still form (Mauger, 1972a). On the other hand, their formation is clearly not prevented in dorsal skin of the scaleless mutant (Goetinck and

Sekellick, 1970) by those epidermal deficiences (Sawyer and Abbott, 1972) which preclude the development of placodes. The wide occurence of this type of structure and its relative independence of the important events leading up to primordium formation point to some general rôle in maintaining tissue architecture under particular conditions and suggest that, if it is involved specifically in feather morphogenesis, it may be secondarily adapted to this purpose. In fact, despite the work which has gone into describing these bundles, their function remains a mystery and can only be guessed from their relation to other parts of the system. Palade and Farquhar (1965) have noted that the bundles appear to enter and intertwine with the materials of the basement membrane, while at their base they are entangled in the collagen matrix of the dermis. Kallman et al (1967) observed that the bundles are often associated with dermal cells of unusual orientation which lie perpendicular to the skin surface, and that groups of filaments branch laterally to entwine with the processes of these dermal cells. These relationships have led to the suggestion that the bundles hold dermis and epidermis together, an hypothesis which is embodied in the term 'anchor filament bundles' used by these authors.

The Importance of Dermal Histogenesis: Experimental Evidence

Despite the fact that the capacity to form feathers is determined early in development, its expression is dependent on the embryonic environment until shortly before morphogenesis of the pteryla begins. Straus and Rawles had shown in 1953 that lateral plate mesoderm and its overlying ectoderm from $2\frac{1}{2}$ to 3 day-old embryos develops feather structures when cultured on the CAM. However, within the next decade attempts to culture skin in chemically-defined, or supplemented, media in vitro showed that

feather development would only proceed in skin explanted from embryos more than $5\frac{3}{4}$ to $6\frac{1}{2}$ days old (Sengel, 1958; Bell, 1964; Wessells, 1965). The time of the threshold varied with culture conditions. An analysis of the requirements in one particular culture system is reviewed by Sengel (1976b).

Wessells (1965) was the first to note that the change from dependence on the embryonic environment to independence is accompanied by the transformation of loose mesenchyme into dermis at the presumptive site of the initial row, and he suggested that the latter is a necessary condition for the former. This correlation was confirmed and extended by later authors (for example, by Ede et al, 1971) who noted that the transformation spreads through the pteryla in advance of primordium formation. The formation of dense dermis is also observed prior to the formation of Avian scales (Dwyer, 1971: quoted by Sawyer, 1972a). The formation of Reptilian scales is preceeded by an increase in the thickness, if not in the density, of the subectodermal mesenchyme (Maderson, 1965).

Experimental evidence obtained by Sengel's group (reviewed by Sengel, 1971) further supports the conclusion that the early development of dermis is crucial for feather formation. A variety of treatments - X-irradiation, excision of the somites or of the neural tube, and hydrocortisone injection (for details of these treatments and their possible action, see Sections 2.4 and 2.5) - each presumably acting at a different point in development, all prevent the formation of dermis in the pteryla and all prevent the formation of feather primordia in the same region. This evidence must, however, be treated with caution, because none of the treatments applied are specific in their effect on the development of the dermis.

Sengel has further suggested that the attainment of a high cell population density *per se* is a sufficient condition for the development of skin which can form feathers. (Again, the evidence is reviewed by Sengel, 1971.) Experimentally created fusion between somatopleural and splanchnopleural tissue gives rise to an unusually high cell population density in the presumptive ventral apertium and results in the formation of a supernumerary feathered area (Keiny and Sengel, 1964). The extent of feather formation in these areas was roughly proportional to the effectiveness of the methods used to 'fuse' the tissues. Dhouailly (1978) has observed that even the extra-embryonic somatopleure will form feather filaments, apparently as a result of an artificial increase in mesenchymal cell population density. Again, however, none of these treatments are specific in their effect and the conclusion must therefore remain tentative.

Although an increase in cell density in the mesenchyme may be an important primary step towards feather formation, molecular changes in the mesenchymal, extracellular matrix may also be of crucial importance. The *in vitro* differentiation of epidermis in 6 day-old thigh skin as a keratinized epithelium shows a strong correlation with the accummulation of glycoproteins and hyaluronic acid in the developing dermal matrix (Carinci, Stabellini, and Bechetti, 1978). Suggestive, though incomplete, evidence (reviewed by Carinci *et al*) points to a possible rôle of the dermal matrix in promoting epidermal differentiation. It is interesting to note that the direction of development of thigh skin can be controlled according to the presence of serum or embryo extract in the culture medium (Carinci *et al*, 1978), for a comparison of the media used by different authors to culture feather-forming skin suggests that

similar differences in composition govern whether or not dorsal skin, explanted before primordia are present, will form feather structures in vitro. It is therefore possible that similar molecular changes in the forming dermal matrix are crucial to the development of the competent, feather-forming skin.

3.2 Necessary Tissue Interactions

The evidence discussed in this subsection indicates that tissue interactions are necessary for the initiation and development of primordia. These come into the general class known as secondary inductive interactions, 'induction' being defined, in this case, as a crucial interaction between two tissues which permits or stimulates one of them to embark on a specific path of development.

The general approach used to reveal and study induction is to examine the development of experimental tissue combinations as described in Section 2.2. However, it must be noted, in the cases described here, that we are interested in the whole development of the organ primordium, not merely the formation of a visible pattern. Since only morphological criteria have been used to assess the development of tissue combinations in the following examples, conclusions regarding the molecular aspects of the developmental outcome cannot be made.

The limitations of the tissue combination approach applied to the skin system are particularly severe. Wessells (1965) has pointed out that major rearrangements of cells and tissues often follow recombination of the dermis and epidermis before normal morphogenesis begins, and consequently the technique cannot define the stage of inductive activity, but can only determine if the combined tissues have the capacity for morphogenesis. A further

fundamental difficulty in applying the concept of induction here is that of asigning active and reactive rôles to the skin tissues. This follows from the formal symmetry of the experimental system: a positive result involves the development of both tissues in concert, and depends on the inherent properties of both tissues, because competence to react is as crucial as activity. Moreover, there is no reason to suppose that one tissue may not play both active and reactive rôles.

For these reasons, and because the mechanisms of the tissue interactions themselves are not understood (Sengel, 1976b; Saxen, 1977) evidence from tissue combination experiments provides no direct insight into the mechanics of pattern formation and morphogenesis. However, to emphasise that the morphological changes in the developing primordium, which will be discussed later, depend crucially on these interactions, it is necessary to briefly review the main evidence for their importance. The extensive literature on the subject has been recently reviewed by Sengel (1976a and b).

Examining first the initiation of primordium morphogenesis, we may begin by considering the importance of the dermis. It is clear from the preceeding discussion of the importance of dermal histogenesis that regional properties of the dermis play a crucial part in determining the direction of epidermal development. Tissue combination experiments provide further evidence of the importance of these interactions. A wide variety of epithelia which would never normally form feathers do so in combination with feather-forming dermis: for example, epidermis from the midventral apertium (Sengel, Dhouailly, and Keiny, 1969), chorionic ectoderm (Kato and Hayashi, 1963), and proamniotic epithelium (Mizuno, 1972); other examples are provided by Sengel (1976b). In contrast to the

dermis from pterylae, the mesenchyme from apertia does not participate in feather formation, even in combination with dorsal epidermis. Evidence that the dermis defines the pattern close to the time of morphogenesis (see page 13), further implies that epidermal development depends on the local properties of the dermis at the prospective primordium site. However, again, there is no evidence relating these properties to local dermal morphogenesis.

The details of these interactions have been examined by Sengel (1958, 1964, reviewed 1976a). On the gelled agar medium of Wolff and Haffen, supplemented with embryo extract from 9 day-old chicks, combinations of tissues of different ages were tested. From the combined results of several experiments Sengel has concluded that the dermis from skin with one to three rows of primordia possesses a transient inductive activity which instructs the epidermis to initiate morphogenesis. Interpretation of these experiments is complicated by the fact that the results are in terms of the developmental state of the whole skin and therefore cannot be related to the local properties of the dermis at prospective feather sites. Moreover, in other culture conditions, the dermis shows a continuous capacity to form feathers in combination with different ages of epidermis (when cultured on the CAM (Rawles, 1963) or in completely defined, protein-free Waymouth's medium in vitro (Wessells, 1965)). Rawles and Wessells concluded that the hypothesis of transient inductive activity is no longer tenable. Certainly, till more is known about the effects of different culture environments on dermal development, the results of Sengel's experiments are difficult to interpret. The experiments of Carinci's group (see page 49) promise some insight in this direction.

The epidermis also plays a crucial part in initiating morphogenesis. This is clear from the development of the scaleless mutant. Epidermis in the homozygous scaleless mutant fails to form either feather or scale placodes, though as far as may be judged its morphology is otherwise normal (Goetinck and Sekellick, 1970; Sawyer and Abbott, 1972). Goetinck and Abbott (1963) and Sawyer and Abbott (1972) have shown, by reciprocal combination of tissues from mutant and normal skin, that the defect resides in the epidermis, while mutant dermis participates in feather or scale formation in association with normal epidermis. However, there is no firm evidence of the primary effect of the mutation. The point of interest here is that condensations and the normally associated dermal cell patterns are absent in the mutant. The dermis of intact mutant skin undergoes a general densification (Goetinck and Sekellick, 1970). The same is true of normal dermis in combination with mutant epidermis (Goetinck and Abbott, 1963). Clearly, interaction with normal epidermis is required for dermal morphogenesis.

It has also been found that well-formed epidermal placodes can cause the initiation of dermal condensations in certain tissue combinations. This has been noted by Linsenmayer (1972) and Novel (1973), but the most striking example is provided by Sengel (1958). Epidermis from skin with several rows of primordia was placed on the underside of whole skin, that is, in contact with dermal tissue which had never been in intimate contact with epidermis and which would never normally form appendages. Featherbuds formed at the sites of the original placodes of the grafted epidermis so that double-sided skin resulted. Whether or not this phenomenon is related to significant organising activity of the

epidermis, and whether or not the epidermis in intact skin possesses a weak activity of this sort during the initiation of morphogenesis, are unknown.

We can conclude from this evidence that two-way interactions between dermis and epidermis are required for the initiation of primordium morphogenesis.

The evidence also indicates that tissue interactions are important in determining the type of appendage primordium formed.

Feather development appears to be an inherent tendency of any chicken epithelium (Sengel, 1976b): in the most striking example, chicken corneal epithelium forms feather structures when combined with mouse flank dermis (Coulombre and Coulombre, 1971). Feather formation is generally favoured over the development of scales (Sengel and Pautou, 1969). Even in combinations between middorsal epidermis and scale-forming dermis of the tarsometatarsis, feather formation dominates with dermis from young embryos: 9 to 11 day-old tarsometatarsal dermis with 5 to 8 day-old middorsal epidermis forms feathers; later (13 to 15 days development) dermis acquires properties which, in combination with middorsal epidermis, lead to the formation of scales (Rawles, 1963). Even then, however, feather structures were often observed growing from the tips of the scales in these combinations. Similar situations have been described in other zoological classes (Sengel and Dhouailly, 1977).

This evidence might suggest that the dermal appendage-forming influence is relatively non-specific. Certainly there are many examples of combinations where the dermis acts in the initial stages of primordium development without zoological class specificity (see, for example, Section 2.2; see Sengel and Dhouailly, 1977, for review), and within single species there

examples where the dermis acts without regional appendage specificity (for example, 9 day-old tarsometatarsal dermis, see above). However, the situation is not so simple. First, in the case of the tarsometatarsal dermis, appendage specificity is acquired with time (see above). Second, dermis from some regions (for example, the spur region of the tarsometatarsus, and beak (Rawles, 1963)) shows strong regional appendage specificity throughout development: spur dermis, in combination with dorsal epidermis forms only a spur and no other dermis induces the formation of a spur. Rawles (1963) has compared the different regions of the chick dermis in a ranked order in terms of inductive strength, but till more is known about the mechanisms of tissue interaction in different cases these empirical comparisons must be treated with caution. Finally, evidence from combinations of dermis and epidermis from different zoological classes has been interpreted to indicate that the dermis exerts a class-specific influence on the late stages of primordium development, for these combinations generally produce only abortive appendage primordia (Dhouailly, 1975, Sengel and Dhouailly, 1977). However, it is not clear to what extent the failure of development is due to the lack of specific interactions or to gross developmental incompatability between the two tissues.

3.3 The Possible Rôle of Dermal Morphogenesis in Pattern Determination

The Temporal Relation of Dermal Morphogenesis to Epidermal Development

Wessells (1965) was the first to emphasise that the epidermal placode forms before the dermal condensation. A more complete study by Sengel and Rusaouën (1968) confirmed this and showed that the placode develops only slightly ahead of the

condensation in the midline of the spinal pteryla, but that successive rows of placodes form more quickly than rows of condensations. By the time three rows of condensations have formed on either side of the midline, placode development is one row ahead; when six rows of condensations have formed, placodes are present about two rows ahead. Sengel and Rusaouën point to the presence of arrays of dermal cells immediately lateral to formed condensations which may intersect at sites of future condensations. However, it is not clear from their account if dermal cells always extend as far as the most recently formed placodes in lateral parts of the pteryla.

Wessells (1965) also noted that changes in the periderm are among the earliest events in primordium formation, pointing out the presence of nuclei which stain intensely with Feulgen stain (which is quantitatively specific for DNA) in peridermal cells near the centre of the emerging placode. If this represents a small group of integrated cells in late stages of the cell cycle, it may indicate that earlier molecular events become locally synchronised at the presumptive site several hours before the placode forms.

Further evidence of early peridermal changes comes from scanning electron microscopy (SEM) studies of the skin (Sengel and Mauger, 1976). Peridermal cells on the anterior face of the primordium show marked transverse orientation in many cases, while those on the posterior slope are generally slightly elongated anterio-posteriorly. Sengel and Mauger found that laterally, "where feather rudiments were as yet undetectable in the living state", there were patches of transversely elongated peridermal cells which corresponded in position to the anterior slope of the presumptive primordia. Again, however, the precise temporal

relation between peridermal patterning and dermal morphogenesis or placode formation is not clear.

In conclusion, the descriptive evidence does not allow us to determine when the primordium site becomes established relative to the time of dermal morphogenesis, and the problem of whether or not the models of Stuart et al and Novel would be possible in this light remains unresolved.

Dermal Patterns

Despite deficiences in the evidence reviewed above, we must examine the morphological and experimental evidence concerning the rôle of patterns of dermal cells and fibres in the initiation of new primordium sites.

The emergence of the first, and each successive, row of feather primordia is described in early reports (Davies, 1889; Holmes, 1935; Watterson, 1942; Goff, 1949) as follows: "a dermal ridge is formed which is then reorganised into a linear series of localised condensations of dermal cells" (Goff, page 449). However, Wessells (1965) and later authors do not describe a ridge, but merely the formation of individual primordia. Stuart, Garber, and Moscona (1972) report that, in the spinal pteryla, the formation of the initial row is immediately preceeded by the appearance of an opaque epidermal stripe which lies along the midline. Beneath this, dermal cells are aligned anterio-posteriorly in a narrow array which is associated with birefringent, apparently extracellular material. Within this array, small clusters of cells appear at the sites of future condensations. There has been no attempt to find out how this initial periodic distribution of cells arises. Instead, this potentially interesting and important question has been ignored and

attention has been focussed on those aspects of development which can be generalised to all primordia of the pteryla.

Stuart and Moscona (1967) were the first to report that as new rows of primordia develop the intervening dermal cells elongate and align to form arrays which join adjacent condensations. These arrays, too, are associated with birefringent, fibrous material which is insensitive to trypsin, but sensitive to collagenase, and stains with Van Geison stain (specific for collagen), suggesting that it contains collagen as a major structural element. Stuart et al (1972) extended these investigations, observing that organised birefringence "extends anterio- and posterio- laterally from the medial papillae (primordia) to the sites of future lateral papillae" (my parenthesis). However, the precise lateral extent of the birefrigence is not clear from these reports. Stuart et al isolated dermis using trypsin and applied a further extensive trypsin treatment to remove cells, leaving behind the presumed extracellular birefringent matrix. No detailed confirmation of the exclusively extracellular nature of this material was reported. Stuart et al note that "a careful examination of these stained (i.e. Van Geison) lattices suggested that laterally, as well as medially, the organisation of their constituent fibres slightly preceeded, in time, the development of papillae and the appearance of elongated dermal cells". These observations led Stuart et al to propose that intersections between arrays define the sites of future condensations and also play a crucial part in the condensation process (see page 38). In support of their model, Stuart et al notethat treatment with collagenase reversibly inhibits both the initiation of new primordia and the formation of a birefringent lattice and arrays of oriented cells. Moreover, both in the scaleless mutant (Goetinck and Sekellick, 1972) and in

embryos treated with hydrocortisone, neither primordia nor arrays of fibres and cells form. However, although the rôle of the fibrous matrix and its associated cells is currently quoted in reviews and texts (Garber, 1976; Sengel, 1976b, 1975), the evidence supporting this is, at present, both controversial and ambiguous.

First, the distribution of arrays of cells in the dermis has been re-examined by several workers. While the presence of cellular arrays between well-formed condensations has been amply confirmed (Wessells and Evans, 1968; Sengel and Rusaouën, 1969; Ede, Hinchliffe, and Mees, 1971), the presence of arrays of cells in the dermis lateral to the most recently-formed condensations is controversial, for, though supported by Sengel (1971), it has been questioned by Wessells and Evans (1968) and by Ede et al (1971). For example, Ede et al, examining whole mounts and tangential sections of the dermis, observed that cells are oriented radially to condensations of the most recently formed row and that "no distinct lattice arrangement of cells connecting neighbouring condensations or preferential distribution of collagen appears before 4 to 6 files (i.e. rows) of condensations are established".

Second, the existence of an oriented fibrous matrix lateral to the most recent condensations or in association with well-defined cellular arrays has not been confirmed by subsequent observations. In attempts to repeat the tryptic isolation of oriented matrix reported by Stuart et al (1972), Wessells and Evans (1968) and Overton and Collins (1976) failed to obtain any lattice of oriented fibres as judged by microscopy. These authors further investigated collagen orientation in the dermis in thin sections examined by TEM. They found no evidence of unique orientation such as might provide an oriented substratum for cell elongation.

Wessells and Evans noted that many structures which have the appearance of fibres in the light microscope are seen to be cell processes when viewed in the TEM and these authors have pointed out that the birefringence observed by Stuart et al may be cellular in origin. This evidence has been criticised by Stuart et al (1972) because TEM cannot, without careful three-dimensional reconstructions or statistical analyses, detect orientations which lie even slightly outside the plane of section. To overcome these problems, Overton and Collins (1976) used SEM to visualise the matrix, in area and depth, in preparations of isolated matrix and in intact dermis. Mid-dorsal skin from both normal and scaleless mutants embryos was examined: the former had between one and five rows of primordia; the latter were matched for age. Overton and Collins found no evidence of orientation anywhere in the normal or mutant skin. The only difference observed between the two types of skin was that the arrangement and fibre diameter of collagen in the mutant appeared more variable than normal. In both cases, fibres in these preparations typically followed curved paths. These authors conclude that their results do not favour collagen orientation as a controlling factor in cell migration. This technique would certainly detect gross alignments in the matrix. However, it remains possible first that the removal of cells distorts the organisation of the matrix, and second that a weak, general orientation passes undetected. This latter possibility is suggested by the observation (Wasoff, personal communication) that alignments of collagen, which are sufficient to orient fibroblasts into striking arrays in vitro (Elsdale and Bard, 1972), show no marked alignment of collagen fibres at similar magnifications in the SEM.

Finally, evidence for the importance of the collagenous matrix in defining the feather pattern is ambiguous because none of the experimental situations intended to test this causal relation are specific in their effect on matrix or dermal arrays. Each is also expected to influence critically other necessary processes and interactions.

First, collagenase destroys collagen, and probably mucopolysaccharides (Bernfield, Banerjee, and Cohn, 1972), and may be expected to have a general disruptive effect on the dermal matrix. In addition, Wessells and Evans (1968) have made particular note of the fact that collagenase destroys the dermal-epidermal interface in feather-forming skin.

Second, the scaleless mutant defect does not directly affect the dermis (see page 53). Assaying collagen synthesis by the incorporation of label into hydroxyproline, Goetinck and Sekellick (1972) investigated the possibility that the epidermal defect in the mutant results in a depression of collagen synthesis in the dermis. However, their results strongly suggested that at least as much collagen is synthesised in mutant as in normal skin. Goetinck and Sekellick, and Stuart et al (1972) (knowing these results) have speculated that the organisation, rather than the synthesis, of collagen in dermal arrays is under epidermal control. There is no evidence for this. It seems more likely that feathers fail to form in the dorsal skin of the scaleless mutant as a result of the same deficiency as is responsible for the failure of scale development in the tarsometatarsal skin. Histological descriptions of scale formation give no indication of cellular arrays (Sawyer, 1972a): rather, evidence points to the importance of the tissue interface. Sawyer and Abbott (1972) noted that the failure of scale formation

in the mutant is associated with an abnormal tissue interface: mutant dermis and epidermis were much more easily separated than were normal tissues during the period when scales normally form. The disruption of the interface appears to affect attachment of both dermis and epidermis to the basement membrane.

Third, reciprocal combinations between the tissues of treated and untreated skin indicate that hydrocortisone acts principally on the epidermis (Stuart et al, 1972). Although Stuart et al suggest that hydrocortisone disrupts the organisation of dermal collagen, there is no evidence that the defect in treated epidermis directly influences morphogenesis of the dermal fibrous matrix. In fact, hydrocortisone affects several other processes which are likely to be intimately involved in the initiation of primordia (see page 39).

This discussion points to the insubstantial nature of the positive evidence for the model of Stuart et al. However, there is equally no positive evidence against the proposal that cellular arrays play a part in determining the feather pattern. Clearly, we need a firm histological description of the dermis lateral to formed primordia and a critical experimental test of the model.

Finally, Novel's model is difficult to examine specifically in the light of the present evidence, especially since the precise nature of the proposed steric interactions is not clearly defined. However, a histological study of cell patterns adjacent to newly formed primordia would be expected to show where such interactions might occur.

3.4 The Early Stages of Primordium Development

Epidermal Placodes

Wessells (1965) provides a good description of the feather placode. The most striking change during placode formation is an increase in height of the basal epidermal cells. These cells lie perpendicular to the skin surface at the centre of the placode. Towards the periphery, where the placode is thinner, cells slant with their apices pointing inward. When the placode first forms, the skin surface remains flat so that the placode centre bulges slightly into the dermis. Anchor filaments - some of which are always found at the centre and periphery - appear to pull the epidermis downward and are associated with well-defined epidermal spurs. Kischer (1968) has described the fine-structure of spurs at this stage. Later, as the dermal condensation develops towards maturity, anchor filaments and spurs disappear from the primordium though they persist elsewhere in the skin. Placode cells continue to elongate, to about twice the height of cells over lateral dermis. Just before outgrowth of the primordium begins, the placode becomes thinnest at its centre and its undersurface becomes concave as the condensation bulges upwards. The whole primordium forms a low dome on the skin surface. Elevation and outgrowth of the primordium must involve local folding of the epidermal sheet which has not yet been described in detail.

The forces which underlie these dynamic changes are not clearly understood. No experimental studies of the process have been made. However, two descriptive observations are relevant. First, peridermal and epidermal cell polulation density is the same in both placode and interplacode regions (Sengel, 1969; quoted by Sengel, 1976b). This implies that the primary cause of cell elongation is not a packing force generated by an increase in cell numbers

in the placode region. It implies, too, that cell elongation involves a considerable increase in volume and surface area. Second, these changes are quite rapid. Stuart et al (1972) estimated, by direct observation of freshly explanted skin in culture, that placodes form in 90 mins.

Some insight into the possible forces involved in cell elongation in the feather placode may be gained by noting evidence relating to other systems. Placodes are important components in several initiating organ primordia, for example, in the developing hair primordia of the rat (Wessells and Roessner, 1965), in the chicken (but not in the lizard) scale primordium (Sawyer, 1972a; Dhouailly, 1975) and in the chicken and mouse lens primordium (McKeehan, 1951; Pearce and Zwaan, 1970). Most of the evidence for the forces responsible for cell elongation comes, however, from studies on the mouse and chick lens placodes where elongation is extreme by comparison with the feather placode, so that extrapolation to the feather system can only be made with caution.

Sengel's estimates of cell density contrast with evidence on the lens and avian scale placodes where cell density increases as cells elongate (McKeehan, 1951; Yohro, 1969). However, in these systems, the evidence suggests that cells may be sequestered into the placode region (McKeehan, 1951; Sawyer, 1972b) as a result of cell elongation due to forces operating on individual cells and - at least in the lens - independent of packing forces (Langman, 1956).

There are two main candidates for cell elongation mechanisms. The first is the assembly of microtubules parallel to the long axis of the cell (Byers and Porter, 1964). However, although they may be involved in cell elongation, microtubules are not immediately responsible for maintaining cell shape and are not required for bending

the epithelial sheet of the lens placode (Pearce and Zwaan, 1970). The second candidate, is contraction of bundles of actin-containing (and perhaps myosin-associated) microfilaments (Ishikawa, Bischoff, and Holtzer, 1969; Trotter, Foerder, and Keller, 1978) which are commonly found at the apices of epithelial cells where contraction might play a part in the observed changes in cell shape (Wessells, et al, 1971; Spooner, 1974). Microfilaments have been implicated in lens placode morphogenesis (Wrenn and Wessells, 1969). In the feather system, contractile forces would, presumably, act isometrically.

Sawyer (1972a) discusses the possible rôle of microtubules and microfilaments in scale placode development, though there is no specific evidence of their involvement. The meagre ultrastructural accounts of the feather placode (Kischer, 1968; Sengel and Rusaouën, 1969; Matulionis, 1970) make no reference to microtubules or microfilaments although these remain possible candidates pending definitive examination. The possible rôle of a passive or active increase in cell volume and surface area has not been considered. Moreover, to account for the initial shape of the whole placode several forces must be involved and their action integrated. Gierer (1977) presents a theoretical interpretation of the integrated morphogenesis of cell sheets, but an experimental approach to the problem is also required. Investigation of the local physical and chemical interactions between the epithelium, basement membrane and mesenchyme promises to be a fruitful avenue of investigation (Hendrix and Zwaan, 1975; Slavkin, Bringas, and Cameron, Le Baron, and Bavetta, 1969). The relation of morphological changes in the placode to the elevation of the whole primordium has not been investigated: it is not known, for

example, if the disappearance of anchor filaments and spurs from the primordium is related to the change in shape of the epidermal-dermal interface.

These morphological changes must also be seen in relation to the cell's metabolism and synthetic activity. Morphogenesis and differentiation may be closely linked to control of the cell cycle. Direct evidence of this is provided by Wessells (1965): before placode formation cells are actively and asynchronously dividing, but immediately the placode forms there begins a transient period of about 20 to 30 hours in which mitoses are rarely observed and few cell synthesise DNA; just before outgrowth, DNA synthesis and mitotic activity are resumed. Periods of localised non-proliferation have been noted in the placodes of avian scales (Sawyer, 1972b), and mouse pelage and (to a variable degree) vibressae hairs (Wessells and Roessner, 1965), though reports differ regarding proliferation in the periderm. Even after mitotic activity is resumed, at least in the scale placode, cells in different parts of the epidermis remain under tight, local control of the cell cycle (Sawyer, 1972b). The significance of this period is not known, but it is possible that it reflects a basic change in the molecular syntheses of the epidermis: a similar non-proliferation phase immediately precedes the onset of intensive keratin synthesis in the developing feather filament epidermis around 12 to 13 days development (Kemp, Dyer, and Rogers, 1974).

There has, however, been no systematic study of other molecular syntheses during feather placode formation: evidence indicating changes in the synthetic activities of the cells comes from disconnected observations which cannot be related at present to important developmental events (Ben-Or and Bell, 1965; Hamilton, 1965; Wessells, 1965; Kischer, 1968; Matulionis, 1970;

Ede et al, 1971).

In relation to the significance of these morphological and biochemical changes for other aspects of primordium formation, it is perhaps surprising to find that placode formation is not strictly essential for epithelial development or for certain associated aspects of dermal morphogenesis. The normal development of vibrissae involves placode formation in some cases and not in others (Wessells and Roessner, 1965). Scale dermis at the ridge stage (Sawyer, 1972a) can induce the formation of scales in chorionic epithelium in a series of developmental changes which, though involving cell elongation, does not involve the formation of placodes as such (Kato, 1969). Finally, dermal condensations, albeit of irregular size and shape, develop in a few instances when scaleless mutant dorsal skin is cultured on the CAM, yet no placodes are formed nor does the epidermis thicken generally (Goetinck and Sekellick, 1972). Though these instances are clearly the exception rather than the rule, they do emphasise that caution is required in drawing too close a connection between obvious morphological changes during placode formation and the essential developmental processes.

The view which emerges from this account of placode formation is of a poorly-understood process, described, but not analysed in the literature.

Dermal Condensations and Arrays

A. Morphological Evidence

Condensations of mesenchymal cells are formed in a variety of organ primordia (for example, in primordia of the developing duodenum and the submandibular gland of the rat (Mathan, Hermos, and Trier, 1972; Cutler and Chaudhry, 1973) and of the

limb cartilage and the scleral ossicles of the chick (Thorogood and Hinchliffe, 1975; Hale, 1956). They are not, however, common to all skin appendages: dermal condensations are formed in vibrissa and pelage hair primordia (Hardy, 1951; Wessells and Roessner, 1965), but not in the Reptilian scale (Maderson, 1965; Dhouailly, 1975) or in the pre-ridge stages of the chicken scale primordium (Sawyer, 1972a), though in the latter case small condensations of dermal cells form immediately beneath the apical folds of the developing scale ridge (Dwyer, 1971, quoted by Sawyer, 1972a). Among other condensations, that of the feather is unusual in its association with striking patterns of mesenchymal cell orientation.

Wessells (1965) noted that the first sign of a feather condensation is the appearance of a small group of dermal cells with characteristic morphology below the placode. In time, more cells in this region change their morphology and become densely packed in a well defined, lens-shaped condensation about 235 μm in diameter and 47 μm thick. From counts of nuclei in transverse sections, Wessells estimated that the cell population density increases in condensations, so that in mature condensations, before the outgrowth to feather buds, cell density is about twice that observed in dense dermis. In addition, the condensation becomes thicker than the surrounding dermis: its base bulges into the subdermal mesenchyme from the early stages of development; closer to the time of primordium outgrowth, the apex of the condensation becomes elevated above the level of the intercondensation dermis (Fig.1)(Wessells,1965; Ede et al, 1971). Cells in the central mass of the condensation are rounded and, in comparison to other dermal cells, typically appear to have larger, spherical nuclei with conspicuous nucleoli, and have more prominent cytoplasmic regions (Wessells, 1965; Matulionis, 1970). In

contrast to these, cells at the periphery of the condensation are spindle-shaped, with elongate nuclei: they show particular patterns of orientation in the plane parallel to the skin surface (Ede et al, 1971). Many of these cells lie parallel to the circular boundary of the condensation, while beyond this cells are oriented radially in the early stages of development, later (according to Ede et al, 1971) becoming aligned towards neighbouring condensations. According to Sengel (1971) arrays are present between condensations, from the outset of condensation development.

Whatever the time when they first become apparent, it is clear that arrays become progressively more organised as development of condensations proceeds (Wessells and Evans, 1968; Ede et al, 1971). The evidence concerning the morphology and structure of arrays has already been discussed (page 58). The dermis between arrays is sparsely populated with stellate and spindle-shaped cells which lack consistent orientation (Wessells and Evans, 1968; Ede et al, 1971).

Several descriptive studies indicate an increasingly close association between the apex of the developing condensation and the basement membrane. Sengel and Rusaouën (1969) noted that minute protrusions of mesenchymal cells come close to the basement membrane. These are already visible in the TEM throughout the presumptive dermis in the 5 day-old embryo, but become more numerous and approach closer to the epidermis within the primordium in 6 and $7\frac{1}{2}$ day-old embryos. Kischer (1968), examining the fine structure of the primordium, reported that mesenchymal cell processes approach sufficiently close to epidermal spurs to suggest actual connection. Kischer also noted that minute protrusions of epidermal cells are surrounded in a cup-like fashion by mesenchymal processes.

However, it is not clear from Kischer's account if these associations are confined to the developing primordium. In none of these cases is there clear evidence of direct contact between epithelial and mesenchymal cells such as has been reported in some other interacting tissue systems (Mathan, Hermos, and Trier, 1972; Cutler and Chaudhry, 1973; Slavkin and Bringas, 1976): in each case, contact is via the basement membrane. According to Sengel and Rusaouën, the minimum distance between epidermis and dermis at $7\frac{1}{2}$ days development is about 100 nm. The significance of this close association has not been examined.

The basement membrane is about 200 nm thick over most of its length, though it is locally thickened in places where dermal cells approach particularly close (Sengel and Rusaouën, 1969). It is an electron-dense layer comprising fine, fibrous material embedded in an amorphous matrix and separated from the plasma-membrane of the epidermal cells by an electron-transparent gap of approximately uniform width (Matulionis, 1970). According to Kischer (1968), thin cell processes are consistently to be found enshrouded in basement membrane material at all times during the period of primordium development.

B. Biochemical Evidence

These morphological changes are accompanied by changes in the metabolic and synthetic activities of the cells, but the evidence relating to these molecular aspects of development is mainly descriptive so that their morphogenetic significance remains unknown.

The most striking of these metabolic changes occurs in the central mass of the condensation where all but a few cells cease DNA synthesis (as judged autoradiographically) and there is a conspicuous absence of mitotic figures in histological sections

(Wessells, 1965). This non-proliferation phase correlates closely with the early stages of dermal condensation: it begins as soon as the condensation appears and lasts 20 to 30 hours, concurrently with the non-proliferation phase in the placode; moreover, similar phenomena occur in the condensations of the pelage and vibrissa hairs of the rat (Wessells and Roessner, 1965), but there is no cessation of DNA synthesis in the non-condensed dermis of the chicken scale (Sawyer, 1972b). However, as in the placode, the significance of non-proliferation is unknown, and the mode of control over the cell cycle and the stage at which arrest occurs remain to be examined. Proliferation is resumed immediately before outgrowth of the primordium.

Several workers have noted an increase in alkaline phosphatase activity in the dermis, which appears to be closely linked to the process of condensation, (Koning and Hamilton, 1954; Goetinck and Sekellick, 1970; Ede et al, 1971). Koning and Hamilton noted that the greatest activity is found immediately below the epidermis, decreasing gradually through the condensation to the surrounding dermis. Studies using a varienty of inhibitors of the enzyme strongly suggest that inhibition of activity is correlated with the prevention of condensation (Hamilton and Koning, 1956; this evidence has been reviewed in detail by Hamilton, 1965). Conversely, inhibition of the condensation process using agents such as penicillamine and β-aminoproprionitrile which disturb the cross-linked structure of collagen does not inhibit alkaline phosphatase activity, but rather, activity increases uniformly throughout the dermis, (Goetinck and Sekellick, 1970). It appears, therefore, that this enzyme is necessary for the condensation process. Its precise rôle in this process is not, however, clear - the enzyme

catalyses the release of phosphate from ester linkages in monophosphates such as glucose-6-phosphate. Hamilton (1965) has suggested, and provided some evidence of, a correlation between the activity of the enzyme in the dermis and the presence of RNAase-sensitive basophilic material in the placode. He suggested that these molecular changes are directly involved in the tissue interactions which occur around this time. However, the present data is limited to these easily assayable molecules. Until a wider study of metabolism and synthesis associated with morphological development in the condensation and placode is made, the relevance of these observations to the essential processes of primordium development will remain obscure.

During condensation development, there also emerges a particular pattern of matrix synthesis which clearly reflects polarity in the biochemical organisation of the condensation. Collagen is gradually laid down in the base of the condensation parallel to the skin surface in quantities sufficient to be detected using Van Geison stain (Ede et al, 1971). Almost as soon as condensations are formed, acid mucopolysaccharide deposition (assayed using alcian blue stain and $S^{35}O_4$ incorporation) is markedly increased over that in the surrounding dermis. Deposition is observed at first throughout the condensation, then preferentially at its base (Ede et al, 1971; Sengel, Bescol-liversac, and Guillam, 1962). These changes in molecular composition have not, however, been related to any changes in the basal structure of the developing condensation, nor has there been any histochemical study of the basement membrane during feather primordium development so that we do not know what is the molecular basis of the morphological signs of dermal cell interaction with the membrane described above.

With regard to the development of dermis between condensations, there is evidence of considerable depostion of collagen, as assayed by transmission electron microscopy (Wessells and Evans, 1968) and histochemically (Ede et al, 1971). Ede et al found that mucopolysaccharide deposition, in contrast, is less intense and occurs diffusely throughout the dermis. The significance of these molecular syntheses in relation to the structural organisation of cellular arrays is not known. The evidence regarding the organisation of these molecules in the dermal matrix has already been discussed (page 60).

C. Experimental Evidence on the Dynamics of Morphogenesis

The descriptive evidence thus far reviewed gives a picture of increasing structural order and biochemical differentiation in the dermis as cells become packed within condensations and oriented around and between them. What forces and aspects of cell behaviour are responsible for building condensations and arrays, and what is the dynamic relation between these two major structures? Experimental attention has been focussed mainly on the question, how do cell numbers increase in condensations?

Wessells (1965) was the first to investigate this problem by examining the rôle of cell proliferation in the condensation process. Wessells labelled skin of the spinal pteryla with H^3-thymidine for 2 hours at a stage when medial dermis was forming (Stage 28 to 29), then allowed development to proceed in unlabelled medium for 1, 2 or 3 days. DNA synthesis in labelled medium results in the incorporation of radioactive precursor into DNA: subsequent DNA synthesis and cell division in unlabelled medium dilutes the radioactivity in each nucleus. Incorporation of label was assayed by autoradiography of histological sections: silver grains (the

number of which is approximately proportional to the net incorporation of radioactivity) over nuclei were counted. After one day in unlabelled medium, the majority of cells in condensations showed low grain-counts, while the majority in the surrounding dermis showed high grain-counts.

These results led Wessells to conclude that the dermal condensations form as a result of a brief phase of locally increased mitotic activity.

Individual condensations form rapidly (Wessells estimated, in 6 to 12 hrs.); too rapidly to allow passage through more than one cell cycle. Moreover, the increase in population density is not great and could be accounted for by a single replication of each presumptive condensation cell. Thus, Wessells concluded that the increase in proliferation may involve only one round of mitosis and that the impression of 'denseness' given by histological sections is largely due to an increase in cytoplasmic volume and close packing of the cells. These morphological changes presumably follow immediately on the tail of replication, for as soon as the condensation becomes clearly visible the phase of non-proliferation begins in the condensed core.

Wessells also noted, however, that cells immediately surrounding the core show a high level of DNA synthetic activity in specimens fixed immediately after labelling, and that mitoses are especially frequent in this region. A similar observation was made by Wessells and Roessner (1965) with regard to the cells around quiescent condensations of vibrissa and pelage hair primordia in the mouse. Wessells (1965) therefore suggested that proliferation of these peripheral cells may also contribute to the attainment of the final cell mass in each condensation. He labelled skin containing

quiescent condensations using tritiated-thymidine, then cultured it in unlabelled medium. The core of unlabelled cells maintained its integrity and was 'invaded' by few labelled cells; the lower and outer part of the condensation, in contrast, contained a very high proportion of labelled cells, often observed to show markedly fewer grains than intercondensation cells. Thus, it seems that the dermal condensation is remarkably stable in its core and is added to by the active division of cells in the immediately surrounding dermis. Similar observations and conclusions were made by Wessells and Roessner (1965) as regards the condensations of vibrissa and pelage hair primordia.

One source of difficulty in interpreting these results is the indirect way in which the location of the initial dividing population of cells forming the condensation core is assessed. Thus, as Stuart et al (1972) have noted, cells comprising the core may have divided in the surrounding dermis then migrated into the condensation. Stuart et al therefore tested Wessells conclusion concerning the localisation of cell proliferation by assaying mitoses directly in skin which had been treated for about 2 hrs. with colchicine to arrest mitoses and thus increase the number available for counting. They made a profile of the number of mitoses along the length of the initial thoraco-lumbar row and in the adjacent, as yet undifferentiated, dermis. Although no consistent pattern of local accummulations of mitoses was observed, the resolution of this analysis is not high and the profiles obtained were complex. However, Stuart et al concluded that condensations do not form primarily because of increased proliferation at the sites of prospective, or developing, primordia. There is, however, no justification for this conclusion. Even allowing that there were

no local accummulations of mitoses, allowance was not made for local variations in population density in the dermis (for example, by estimating the percent mitoses), so that no assessment of differential levels of proliferation is possible. Moreover, the dynamics of proliferation cannot be estimated from the numbers or proportion of mitoses alone (Hoffman, 1949). Thus, a direct analysis of cell proliferation in forming condensations remains to be done.

There remains a further gap in the evidence concerning the rôle of cell proliferation in the increase in cell numbers below the placode. The crucial question is to what extent does proliferation of cells below the placode contribute to the increase in cell numbers during the formation of the condensation. It is possible that even uniform levels of proliferation could contribute significantly to the growth of the cell population in the condensation if movement away from the condensation was restricted. However, no data are available to estimate this potential contribution.

The alternative to an increase in cell numbers by local proliferation is that cells are added from the surrounding dermis by a process of accretion. One way this might occur is by the movement of cells, individually, towards the condensation site.

Some evidence that cell movement does play a part in the normal process of condensation comes from studies on the talpid^3 mutant. In this mutant the mesenchymal cells are more adhesive and show a lower net mobility in culture than control cells (Ede and Flint, 1975a and b), but the ectoderm shows no morphological abnormality in the integument (Ede, Bellairs, and Bancroft, 1974). Ede et al (1971) showed that, in the talpid^3 skin, condensations are less sharply defined than normal; their edges merge with the

surrounding dermis which maintains a higher density of cells than in controls and contains no clearly defined intercondensation arrays. The implication is that a certain degree of cell movement is necessary for the normal development of dermal arrays and condensations. However, although condensations in the mutant do not have an entirely normal structure, they are clearly visible and their internal structure shows changes in cell morphology and packing similar to those found in normal primordia. This implies that several essential features of condensation development are not severely impeded in the mutant. Whether the effects of the mutation on cell movement and orientation are insufficient to prevent the involvement of these processes in condensation, or whether other processes play a key and independent rôle, remains unanswered. Finally, the effect of the mutant defect on cell proliferation in the vicinity of the condensation was not investigated: differences in proliferative behaviour between talpid and normal cells in the limb-bud (Ede, Flint, and Teague, 1975) suggest that a normal pattern of cell proliferation in feather dermis cannot be taken for granted, so that conclusions from these observations regarding the relative rôles of division and movement must remain tentative.

One way in which cell movement might contribute to the condensation process is if it were channelled towards condensations by a fibrous matrix or the mutual alignment of cells within intercondensation arrays, as suggested by Stuart et al (see page 38). However, the lack of positive evidence for this has already been emphasised. Some suggestive, though not conclusive, evidence against this possibility has been put forward by Wessells and Evans (1968) who made a particular examination of the polarisation of cells in arrays based on the distribution of organelles described by

Trelstad, Hay, and Revel (1967). Trelstad et al (1967) and more recently Trelstad (1977), have noted a polarisation of organelles in mesenchymal cells which consistently seems to reflect directional morphogenetic movements: the golgi body is generally located at the presumed trailing edge of the nucleus. Wessells and Evans found no evidence of consistent polarisation in adjacent cells in an array and many cells showed no internal signs of polarisation, suggesting that these cells are not involved in active migration towards condensations. However, either a much more firm analysis of the relation between net cell movement and the probability of organelle polarisation in individual fixed cells, or a direct examination of cell movement in arrays, would be required to verify this point.

The mechanism by which the arrays themselves form is not known. As Wessells and Evans point out, the whole question of the dynamic relation between arrays and condensations in the feather system is poorly understood and the evidence does not distinguish between three possibilities: first, that the development of condensations depends on the formation of arrays; second, that the reverse is true; and third, that they develop independently, from a common cause. As has been emphasised above, a description of the morphological relation between the earliest discernible condensations and the laterally extending arrays is a prerequisite to any investigation of these possibilities. Meanwhile, it is relevant to note that while dermal condensations contribute to the final structure of the definitive feather, arrays are transient structures: cell orientation between, and surrounding, condensations is lost as the skin grows and primordia become separated (Ede et al (1971) note that this occurs around 9 to 10 days development, and dermis

surrounding condensations is thinly populated with cells without consistent orientation: the cause or function of arrays thus appears to be limited to the time of condensation formation.

The relative rôles of cell proliferation and movement have been studied in other morphogenetic systems (Abercrombie (1977) provides a short historical review). As regards other organ primordia, short-range centripetal aggregation is involved in pre-cartilage condensations in the chick leg (Ede and Agerbak, 1968; Holtfreter, 1968; Thorogood and Hinchliffe, 1975; Ede, Flint, Wilby, and Colquhoun, 1977) and in mesenchymal condensation leading to kidney tubule formation in the mouse (Saxen et al, 1968): arrays of oriented cells are not involved. In contrast to these systems showing cell movement, the dermal bones of the chick sclera form mesenchymal condensations which apparently arise by an increase in the net rate of cell proliferation (Hale, 1956). However, even in these cases, a complete analysis of both cell proliferation and movement remains to be done. In general, the precise manner in which crucial differences in cell distribution arise during development remains poorly understood, and a study of the process in feather primordia is thus potentially of wide interest.

If cells do move into the region below the placode, some additional mechanism must be responsible for their close packing. This may depend on close-range aggregation caused by specific cell surface interactions as has been suggested by Stuart et al (1972). Moscona (1977) has discussed the possibility that cell surface factors govern cell aggregation and adhesion in higher organisms. However, this possibility in the feather system has not been further investigated, and adhesion between cells in the condensation has not been demonstrated.

Once cells are present in greater density and numbers below the placode than in the surrounding dermis, some mechanism must give this structure stability against the tendency of fibroblastic cells to move outwards from foci of high concentration (Abercrombie, 1970). The data of Wessells (1965) discussed above clearly indicates that the condensation is remarkably stable in this respect and remains so into the phase of feather filament elongation. There are several possible mechanisms by which this stability might be achieved; for example, it could be based on the same cell surface interactions as might cause cell aggregation during the process of condensation. Steinberg (1975) and Harris (1976) have reviewed the evidence - mainly from experiments on the aggregation and sorting-out of cells from mixed cell-suspensions - for and against the possibility that quantitative differences in cell adhesiveness account for the stable distribution of cell types in development. However, the mechanisms which actually operate in the feather primordium, or other organ primordia, are not known.

In experiments similar to those used to test the mutual aggregative properties of cells, skin containing feather primordia has been dissociated, using trypsin, into a cell-suspension which was then cultured on the CAM after a brief treatment in a rotating flask to allow initial aggregates to form (Weiss and Taylor, 1960; Moscona, 1964; Moscona and Garber, 1968). When cells from skin of 8 day-old embryos were treated in this way they formed feather structures on the CAM indicating that the formation of condensations (and epidermal structures) is possible from the intrinsic properties of individual cells and does not absolutely require dissociable supra-cellular levels of organisation as a starting point. The skin used in this study already contained feather structures: it is not clear whether

cells which were previously in condensations reassociated in stable arrangements to form primordia directly, or whether cells lost their differentiated properties and mixed freely to reorganise an undifferentiated skin which then began morphogenesis afresh. Although this is a potentially interesting experimental approach, a more detailed examination of the progress of development in the aggregates would be required before this point could be settled and the results interpreted in terms of the mechanisms of condensation development and stability.

3.5 Conclusions

The present review of the literature points to three problem areas:

1. How do individual epidermal cells of the placode elongate and how are the changes in their shape integrated to produce the form of the whole placode?

2. Do patterns of dermal cells determine the positions of primordia in the feather pattern? In particular, we do not know enough about the extent of dermal cell patterns lateral to formed condensations in relation to the lateral extent of placode development at already established primordium sites. In addition to this gap in the descriptive evidence, a critical experimental test of the model of Stuart et al (1972) is required.

3. How do dermal condensations form? The relative rôles of cell division and movement in establishing an increase in the number of cells below the placode, and the forces which stabilize the developing condensation are not understood.

4. LINES OF FURTHER INVESTIGATION

The evidence presented in the preceeding review of the literature has allowed us to formulate particular questions regarding on one hand the way in which the time and position at which primordia develop are determined, and on the other hand the mechanisms responsible for primordium morphogenesis. These questions cover a wide problem area and I have selected for experimental study two key problems which can both be initially approached by investigating the morphogenesis of the dermis in the period immediately before and during the earliest stages of primordium formation.

The first of these problems is whether the position and time of formation of a primordium is governed by dermal cell patterns extending laterally from existing primordia. This problem has been approached in three ways. First, to fill a gap in the current evidence, a description is given of how far cell patterns extend into the lateral dermis before primordia begin to develop. Second, the way in which pattern formation in lateral skin depends on continuity with medial skin is investigated in a series of experiments in which skin is cut in different ways and its development observed in culture. Third, the similarities and differences between the feather patterns formed in vivo and in culture have been examined to provide further insight into the basic pattern forming process.

Evidence from these approaches is presented in Chapter 4, where it is shown that the lateral extension of dermal cell patterns from established primordia plays no necessary role in initiating, and defining the position of, primordium development. The evidence presented also relates to some of the constraints which do determine the feather pattern. On one hand, the positions of individual

primordia are not irreversably fixed about one day before morphogenesis, but appear to be influenced by local constraints generated by surrounded primordia. On the other hand, the evidence points to the importance of organisation which exists within the skin before primordium morphogenesis. In particular, it is shown that the time at which primordium morphogenesis begins is preset over most of the pteryla before the first row forms. Furthermore, the evidence suggests that the anterio-posterior positions of primordia may be partly determined before the first row forms.

The pre-existing organisation in the skin is further considered in Chapter 6 where the feather pattern is discussed in relation to the patterns formed by other organ primordia.

The second problem is how the dermal condensation is formed; in particular, what are the relative rôles of cell movement and proliferation and what forces are responsible for the structural stability of the condensation? To investigate these questions it has been necessary to look more carefully at the morphology of the developing dermis in vivo. At the same time, the dynamics of the process have been investigated in cultured skin using three approaches: 1. Quantitative examination of the contribution of cell proliferation to the condensation process; 2. a brief examination of cell movement in the dermis by time-lapse filming; 3. a series of experiments using colcemid as a tool to further examine the rôles of cell division and movement during condensation morphogenesis. Finally, the question of why the condensation is stable has been investigated by looking at how the skin containing developing primordia comes apart under gentle dissection treatments.

These approaches are described in Chapter 5. Evidence is presented which indicates that the increase in cell numbers within

a condensation is not due to an increase in the net rate of cell proliferation and implies that cells move into the condensation. The evidence suggests that cell migration along dermal arrays does not make a major contribution to the condensation process and alternative modes of cell recruitment into the condensation are discussed. Finally the results indicate that strong attachment of dermal cells to one another and to the basement membrane in an expanding region at the centre of the developing condensation may be responsible for the stability of the structure.

The mechanisms of mesenchymal condensation in the feather primordium are discussed in Chapter 6 in relation to the morphogenesis of other organ primordia.

CHAPTER 2

GENERAL MATERIALS AND METHODS

1. MATERIALS

1.1 Eggs

Eggs were supplied by Easter Howgate Farm, Midlothian. These were descended, through several generations, from an initial Rhode Island Red X White Leghorn cross subsequently crossed with White Leghorn. After storage at cool room temperature for up to 6 days, eggs were incubated at 38.5°C in a humidified atmosphere for the required period.

1.2 Solutions

Dissection and Culture Media

Skin was dissected and handled in Dulbecco saline A (Oxoid) which is free of calcium and magnesium.

F10 medium (Flow Laboratories) was used for culture of whole skin or fragments. This was supplemented with serum from new born calves (10%; Flow Laboratories), glutamine (146.5 mg/l), and ascorbic acid (10 mg/ml) (except in experiments to examine cells moving out from the boundary of whole skin or fragments). Antibiotics (Glaxo) were added: penicillin (100 units/ml), streptomycin (100 µg/ml), and fungizone (5 µg/ml). F10 medium was selected on the basis of availability, although preliminary experiments showed that satisfactory skin development can be achieved using the simpler MEM medium, with added glutamine and serum as above. Ascorbic acid was added to the medium to help preserve tissue integrity: preliminary experiments showed that the addition of ascorbic acid reduces fibroblast outgrowth from the specimen.

Collagen Substrata

These were prepared by the method of Elsdale and Bard (1972) which is based on the fact that acid solutions of collagen are stable at low ionic strength, but form an hydrated lattice when pH and ionic strength are simultaneously and rapidly brought to physiological levels.

Collagen was extracted by treatment of rat tail tendons for one day in 0.5M acetic acid at 4°C. Debris was removed by squeezing the solution through a double layer of muslin. To achieve a low ionic strength, about 300 ml of the strained solution was dialysed, for two periods of 24 hours each, against 4 litres of freshly diluted one-tenth strength MEM medium. The second dialysis was carried out at pH 4.0 (adjusted with HCl). To reduce bacterial contamination to a minimum, the dialysed solution was centrifuged at 17,000 rpm for 24 hours at 4°C. The supernatant was removed, taking care to avoid fatty material on the surface, and leaving 5 to 10 ml residue in each tube. The final, almost clear, solution could be stored at 4°C for up to 5 months. Occasionally, solutions were too thick: these were diluted with 0.5M acetic acid, since thick collagen was found to promote dermal cell outgrowth leading to disintegration of the skin.

Substrata of precipitated collagen were prepared by raising the pH and ionic strength of the collagen solution to that of culture medium. Collagen solution (9 ml), in a sterile tube, was placed in an ice-bath and ten-times strength MEM (1 ml) added to it. Glutamine, ascorbic acid (except as noted above), new-born calf serum, and antibiotics were added to give the same final concentrations as in culture medium. While mixing the solution with a pipette rapidly, but carefully to avoid bubbles, a small volume of

Na OH (0.142M) was added to bring the pH to approximately 7.6 (as judged by the colour of the neutral red indicator in the MEM). The resulting mixture was dispensed without delay, and allowed to set to a gel-like consistency. Aligned substrates were prepared by dispensing 0.5 to 1 ml of the mixture into 5 cm culture dish (Falcon Plastics), then tilting the dish while the collagen set. In the resulting thin layer of hydrated collagen, the general alignment of fibres could be seen under phase contrast illumination to follow the direction of tilt.

Elsdale and Bard (1972) have shown that hydrated lattices prepared in this way contain approximately 0.1% by weight of collagen which exists as interlacing fibrils 50 to 500 nm in diameter, with characteristic 64 nm periodicity.

Colcemid

Colcemid (Ciba) was used at concentrations of 2.7×10^{-5}M, 5.4×10^{-6}M, 5.4×10^{-7}M, and 1.0×10^{-7}M, diluted in Dulbecco saline A.

Trypsin

Trypsin stock solution (0.2%, Difco 1:250, stored at -20°C) was thawed and diluted to one-twentieth strength in precooled (4°C) Dulbecco saline A. This solution was used at 4°C.

2. METHODS

2.1 Dissection

Dissection dishes were prepared by half-filling sterile petri dishes with moulten paraffin wax. The dishes were covered as the wax solidified.

Eggs at the appropriate stage of development were cleaned with methylated spirits and broken at one end using coarse forceps. The embryo was quickly removed to a sterile dish where it was decapitated. Membranes and debris were removed and the stage of the embryo assessed, using the tables of Hamburger and Hamilton (1951), according to the appearance of the beak, head, and limbs. Care was taken during this brief examination not to allow the skin to dry. The body was transferred to a saline-filled dissection dish where it was pinned to the wax through shoulders and tail. While observing the body under a dissecting microscope, a rectangle was cut in the dorsal skin (Fig. 2.1). This area encompasses the width of the spiral pteryla and a length equivalent to about 11 primordia in the initial row in the lumbo-sacral tracts. The rectangle of skin was peeled free using fine forceps, cutting attachments to underlying tissues with scissors to avoid strain in the skin. Still under saline, the skin was laid flat on a sterile glass coverslip. Coverslip and skin were removed from the saline. The tip of the coverslip was applied to the surface of a collagen substratum in an organ culture dish complete with medium, and the skin was allowed to slip flat onto the substratum with a minimum of distortion. If necessary, the skin was gently adjusted to lie flat and undistorted using fine forceps. Technically unsuitable pieces of skin (too thick, because too deeply cut; raggedly cut; or pieces in which dermis and epidermis had separated locally) were discarded. The number of rows of primordia present was counted immediately after explantation of each piece.

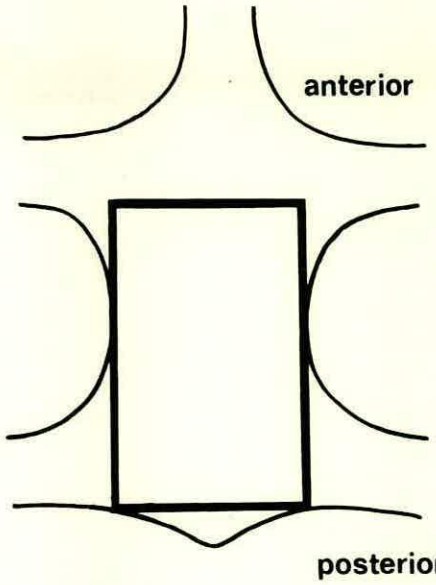


Fig. 2.1 Diagram of the region of dorsal skin used in the present study.

The boundaries of the region are shown by a bold line over the outline of the chick body.

2.2 Organ Culture

Requirements

An organ culture method was designed to meet three principal requirements. First, so that development could be viewed continuously at relatively high resolution using transmitted light, it was necessary to grow the skin on a flat, transparent substratum. Second, since preliminary experiments showed that development ceases in skin submerged to a depth of 1 mm, it was necessary to grow the skin at an air-liquid interface. Third, the method was required to be simple and flexible, so that replicate cultures could be set up and treated reliably with few critical systematic variables.

Method

Hydrated collagen-medium mixture (1 ml) was set within a 1 to 2 cm diameter fence of wire gauze (stainless steel, 60 divisions

per inch, United Wire Works, Edinburgh), in the centre of a culture dish (NUNC, 5 cm petri dish). 2 ml culture medium was added to surround this collagen island. Skin explants were cultured on the collagen surface, at 38.5°C in a humidified atmosphere of 5% CO_2 in air. (See Fig. 2.2)

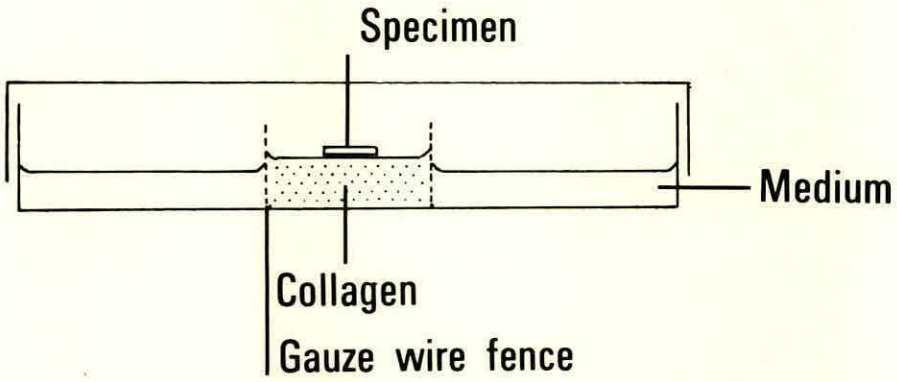


Fig. 2.2 Diagram of the routine organ culture dish.

Collagen acts as a transparent substratum. The fence ensures that the collagen surface is almost flat, with a slight upward meniscus at its edge, instead of the broadly convex surface of an unbounded collagen drop. Medium flows round the collagen and diffuses into the explant from below, without risk of washing over the surface of the explant. Medium can be changed, drugs added, and the explants dissected or removed as required.

Preliminary experiments indicated that culture was simple and reliable providing the pH was maintained approximately at 7.1 to 7.3 (particularly avoiding high pH) and a good flow of air was maintained through the incubator.

2.3 Culture of Skin Fragments on Aligned Collagen Substrata

Skin from the region shown in Fig. 2.1 was removed from embryos at stages 29 to 30 and cut, using a scalpel, into pieces about 1 mm in diameter. These pieces were explanted in about 0.4 ml of culture medium (sufficient to form a thin film over the bottom of the dish) on aligned collagen substrata. Cultures were incubated at 38.5°C in a moist, gassed (5% CO_2 in air) atmosphere in an air-tight box. Next day, when the fragments had settled onto the substratum and cell outgrowth had begun, 2 ml culture medium (without ascorbic acid) was gently added. The cultures were then incubated under the same conditions as used for cultures of whole skin.

2.4 Observation of Living Material

Periodic Observation of Skin in Culture

Developing skin was routinely observed using a Wild dissecting microscope at X 6 to X 30 magnification under both bright and dark field illumination.

Cells at the edge of the explant and those growing on aligned substrata were viewed under phase contrast illumination using a Wild inverted microscope, at X 50 to X 125 magnification.

Occasional use was made of a Zeiss 'Universal' microscope, fitted with Nomarski optics. Nomarski optics reveal detail within the skin because they provide an image of a single plane within the tissue, relatively uninfluenced by structures in adjacent planes. Magnifications of X 78 to X 500 were used. At magnifications in the range X 200 to X 500 a special culture chamber (described in the following Section) was required.

Observations were recorded by photography, using an automatic camera shutter mechanism (Wild, Zeiss). Observations made in

the dissecting microscope were recorded by camera lucida in a few cases where structures could not be adequately recorded photographically.

Filming of Skin in Culture

Time-lapse films were made using either a Wild inverted microscope with phase contrast illumination, or a Zeiss 'Universal' microscope with Nomarski optics. In both cases, a Wild time-lapse apparatus was used in conjunction with a Kodak 16 mm cine camera. Filming was carried out in a room maintained at 37°C. The culture chamber was contained within an outer chamber (Fig. 2.3) into which was passed a continuous flow of CO_2 in air moistened by bubbling the mixed gasses through water and filtered through a cotton wool plug (CO_2 15 to 20 cc/min, air 600 to 700 cc/min; both kept constant in each experiment). The outer chamber was kept moist by placing in it cotton wool, moistened with water.

Filming at relatively low resolution using the Wild microscope was straightforward. The culture chamber was exactly as employed for routine organ culture.

Filming at high resolution, using the 'Universal' microscope presented technical problems demanding a different design of culture chamber.

Requirements for a Special Culture Chamber

High resolution lenses (even long working-distance lenses) require a shorter distance between objective lens and condenser than the distance between the lid and base of a standard petri dish. It was therefore necessary to use a Cooper dish which has a central depression in the lid. Secondly, to avoid unwanted optical interference, the light path must pass through glass, rather than plastic. Thus, the centres of lid and base of the Cooper dish were replaced by

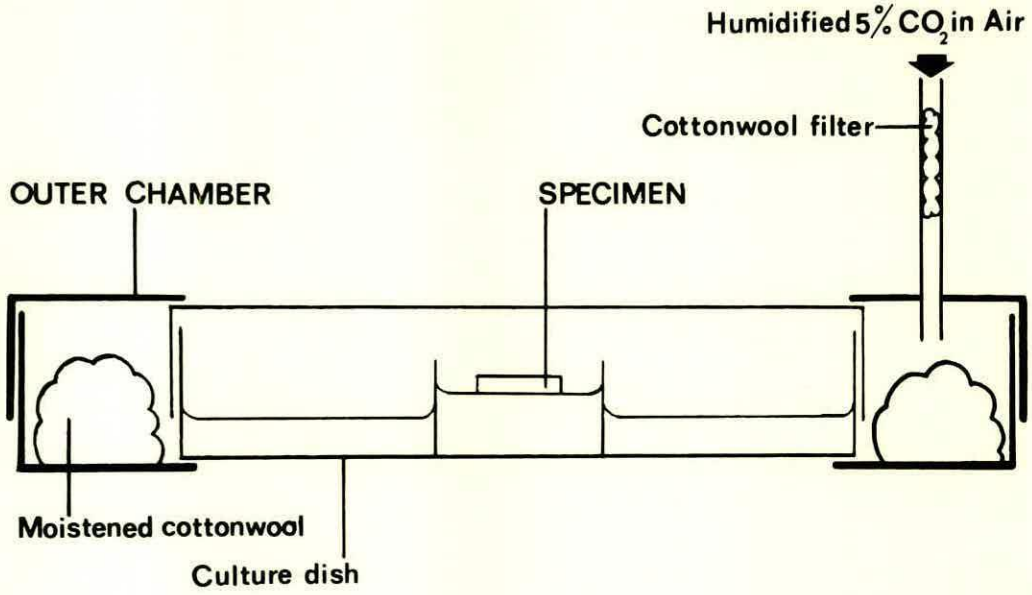


Fig. 2.3 Diagram of the outer chamber used for filming.

The outer chamber is shown in bold lines. Within the chamber, a routine organ culture dish is drawn. This arrangement was used for films made using the Wild microscope. The outer chamber could alternatively accommodate the special culture chamber shown in Fig. 2.4

glass coverslips. Thirdly, the microscope is not inverted. Attempts to culture the skin on the basal coverslip consistently produced condensation on the upper coverslip. The specimen was therefore cultured on collagen on the upper coverslip. This arrangement required that the specimen was kept thoroughly moist: a ring of moistened cotton wool was used for this purpose. (The culture chamber is shown in Fig. 2.4.)

Fabrication and Setting-Up of the Culture Chamber

Circles (15 mm diameter) were cut with a hot scalpel blade in the lid and base of a 5 mm Cooper dish (Falcon Plastics).

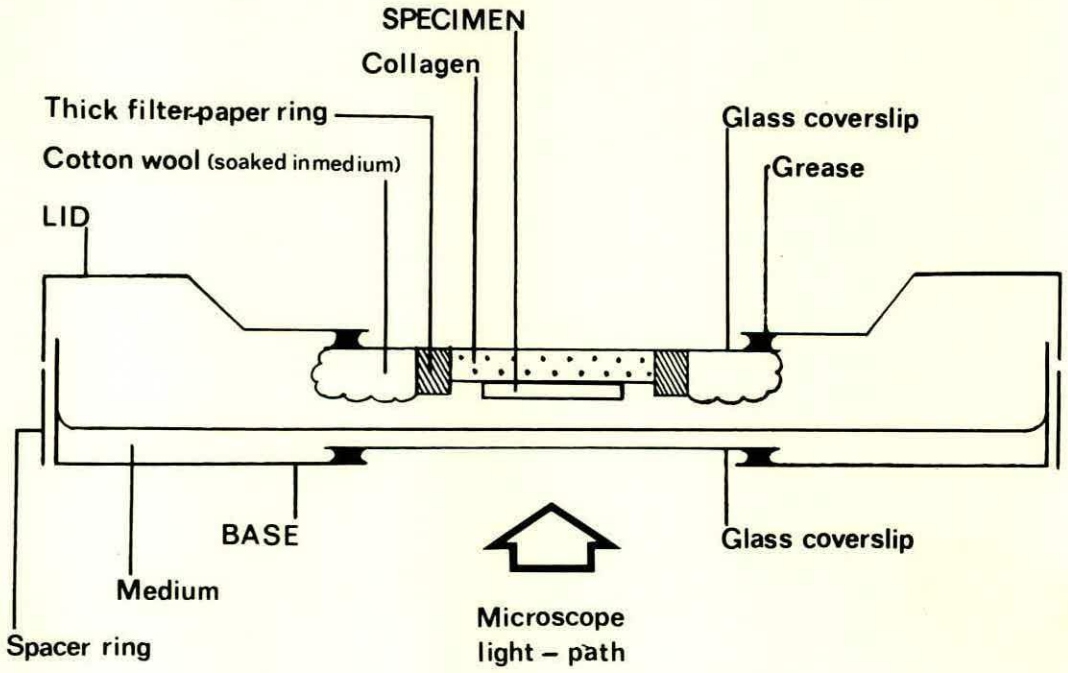


Fig. 2.4 Diagram of culture chamber used for filming with the 'Universal' microscope.

A layer of silicone grease was laid round the inside rim of the holes in the lid and base by squeezing the grease through a syringe. Lid and base were then sterilized by overnight exposure to ultraviolet illumination. Clean, 20 mm diameter glass coverslips were dipped in methylated spirits then flamed. Using asceptic precautions, the coverslips were gently and evenly pressed against the grease on the lid and base to give a seal. To avoid optical distortion, it was crucial that the coverslips lay parallel to the surface of both lid and base. The lid was inverted and a ring of thick filter paper placed on the upper coverslip. Sterile, absorbent cotton wool, moistened with culture medium, was packed around the ring of filter paper. Collagen-medium mixture was prepared and dispensed into the well in the centre of the ring of filter paper. After the collagen

had set, a specimen of skin was placed, dermis downwards, on the collagen. The lid, still inverted, was placed in a large, sterile petri dish and incubated under the usual conditions. The base of the culture chamber was kept in a second, large, sterile petri dish at room temperature. After the explant had attached to the substratum, the base of the culture dish was placed in the base of the outer filming chamber and a spacer ring (cut from a Cooper dish lid) was placed round it. About 1 ml culture medium was dispensed into the base of the culture dish, merely to provide a moist atmosphere during culture. The lid of the culture dish was then quickly, but gently, turned right-way-up and placed on the base so that when the rim of the lid rested on the spacer ring the specimen lay close to, but did not touch, the lower coverslip or the medium in the dish. The lid of the outer chamber was put in place and the whole assembly placed in a holder on the microscope stage in a room at 37°C. The chamber was gassed as described above. After an initial equilibration period (about $2\frac{1}{2}$ hours), the specimen was centred and the optics checked briefly. Optimum resolution is only obtained in this system, using Nomarski optics, with intense illumination. It was therefore important to expose this specimen as briefly as possible to the full intensity of light which would otherwise cause overheating of the skin and failure of development. The optical setting-up and focussing were therefore done under reduced illumination.

Two critical factors govern the success of this method. First, it is necessary to use an optimal thickness of collagen and quantity of medium in the cotton wool: too little, and the specimen develops slowly and unreliably; too much, and medium flows between specimen and lower coverslip, arresting development. The appropriate quantities must be found by trial and error. Second,

regular focussing is necessary, since the collagen dries out slowly and the plane of focus thus changes continuously.

The method is not fully developed, but in the present work the method allowed high resolution filming of cell movement in developing specimens over periods of several hours. With improvements, such as a slow, continuous feed of medium to the specimen, the method may prove useful for longer periods of filming.

2.5 Observation of Fixed Material

Histology: Requirements

It was necessary to provide a flat support so that pieces of skin could be processed and sectioned conveniently and, in particular, so that tangential sections, parallel to the surface of the skin, could be cut. For this purpose, specimens were mounted on filters. Skin to be cut tangentially was mounted on thick, paper filters coated with a thin layer of millipore material on one side (Selection, Dassel, W. Germany). The specimen was mounted on the millipore side. These filters remain flat during processing and, when tangential sections are cut, the knife passes easily through the millipore material. However, the main body of these filters, being composed of coarse cellulose fibres, cannot be cut transversely. For transverse sectioning, specimens were mounted on thin, millipore (GSWPO 1300) filters. Though these sometimes curl slightly in processing through absolute alcohol, they are easily cut transversely.

Histology: Method

Under Dulbecco saline A, specimens of skin were dissected either from the body of the chick, or from the collagen substratum of a culture, using fine forceps and scissors. The specimens were

transferred, flat, onto the surface of filters under the saline. The filters were then withdrawn from the saline, drained briefly - taking great care not to allow the skin to dry - and placed into fixative, skin downward to prevent the skin floating free as it entered the solution. 2.5% gluteraldehyde (TAAB) in cacodylate buffer (sodium cacodylate adjusted to pH 7.4 with 0.1N HCl) was used as a fixative in most cases. Occasional use was also made of Bouins fixative (a gift from the Pathology Department, Western General Hospital, Edinburgh). Specimens were fixed overnight, brought through several changes of 70% ethanol and graded solutions of ethanol in water to absolute ethanol and thence to chloroform. Specimens were cleared in a second change of chloroform overnight and impregnated with paraffin wax (melting-point 56°C) through three changes. They were then imbedded in wax and the blocks trimmed in a manner appropriate for the plane of section required. Sections, 5 to 10 μm thick, were cut using, in early experiments a Cambridge microtome and, in later experiments a Leitz microtome. Sections were mounted on glass slides, stained with Ehrlichs' haematoxylin and eosin, and mounted in DPX.

Sections were examined using a Zeiss 'Universal' microscope, and employing Nomarski optics (slightly offset) to enhance contrast. Photographs were taken using this 'partial' Nomarski effect.

Measurements on Histological Sections

Measurements were made using a Zeiss graticule eye-piece with either a scale graticule (divided into 100 units; Zeiss No. 474007), or a squared graticule (divided into 10 x 10 large, boldly marked squares each subdivided into 4 equal small squares

marked finely; Zeiss No. 474004). The former was used to measure distances; the latter was used to measure area and as a guide in counting cell numbers. Both graticules were calibrated using a scale slide with 0.1 mm divisions. One unit of the scale graticule measured 0.74 μm at X 1250 magnification. One side of one boldly marked square of the squared graticule measured 7.4 μm at X 1250 magnification.

Measurements of structures at the cellular level were made using a planar X 100 objective lens n.a. 1.25 (total magnification X1250). Measurements of distances longer than about 25 μm were made using X 40 and X 16 objective lenses (total magnification X 500 and X 200 respectively).

Where precise estimates of section thickness were required, this was measured using the fine focus of the 'Universal' microscope which is graduated in μm. The difference, in μm, between planes of focus coincident with structures on the top and bottom of the section was taken as the section thickness. Calibration of this measurement is described in Chapter 5.

Scanning Electron Microscopy

Cultured specimens were prepared for scanning electron microscopy after treatment to partially, or completely, remove the epidermis from the dermis (as described for individual experiments in Chapter 5). Under saline, the dermis, with or without the attached epidermis, was dissected free from the collagen substratum and mounted on a small piece of filter paper (Whatman No. 1), then removed from the saline. The filter paper was drained very briefly (taking great care that the specimen remained moist) and placed, dermis downward, into fresh fixative.

Specimens were fixed overnight in 2.5% gluteraldehyde, then washed in two changes (10 mins each) of 5% sucrose in cacodylate buffer (pH 7.4). Specimens were then treated with 1% osmium tetroxide (in cacodylate buffer) for one hour, rinsed in buffered sucrose, and processed through graded solutions of acetone in water: 25%, 50%, 75% and 100%. Three treatments with each acetone solution were applied, lasting 5, 10 and 15 mins respectively. The acetone-saturated specimens were critical-point dried in a Polaron E3000 at 38°C (1200 psi) from CO_2 (critical point 31.5°C, 1100 psi). The specimens, still on filter paper, were stuck with silver paste onto a stub in appropriate orientation. They were coated with gold to approximately 60 nm thickness in an atmosphere of Argon (5 psi) at 5°C using a Polaron E5000 splutter coater. The post-fixation steps, and the critical-point drying and gold coating were kindly done by Mr. Andrew Ross and Miss Carol Gray of the electron microscope service department in the M.R.C., C.P.C. research unit.

Specimens were viewed in an S180 Stereoscan (Cambridge) microscope. Between viewing, specimens were stored in a dessicator.

CHAPTER 3

COMPARISON OF SKIN DEVELOPMENT IN CULTURE WITH SKIN DEVELOPMENT IN VIVO

1. INTRODUCTION

Much of the present study relies on observations on skin developing in culture. It is therefore necessary to give a brief account of how far development in culture resembles development *in vivo*. The following description is confined to observations which enable this comparison to be made.

2. METHOD

Living Specimens

Freshly excised skin was mounted on a collagen support and immediately examined under the dissecting microscope. (54 specimens in all; Stages 29 to 34; at least 5 from each stage: some of these skins were fixed and processed (see below).)

Skin from Stages 29 and 29 + embryos (or as indicated below) was explanted and observed periodically throughout its development in culture. (More than 300 specimens were scanned; 56 from 8 independent experiments, were studied in detail.)

Fixed Specimens

Skin which had developed *in vivo* was prepared for histology in either of two ways: *in situ*, or mounted on filters.

In situ preparations: decapitated bodies were fixed in Bouins, processed for histology, and sectioned transversely or tangentially through the lumbo-sacral skin. (12 specimens in all, from 5 to 8 day-old embryos (Stages 27 to 33); at least 2 from each day of development.

Mounted preparations: Following examination in the dissecting microscope, freshly excised skin was mounted and processed

histologically. (20 specimens from $5\frac{1}{2}$ to 8 day-old embryos (Stages 28 to 33); at least 3 from each day.)

Skin explanted at Stage 29 to 29+ (or as indicated) was allowed to develop in culture then fixed at the required stage and prepared in the same way as, and in most cases in parallel with, freshly excised skin. (22 specimens in all, from 6 independent experiments; containing between 1 and 8 rows formed in culture.)

3. OBSERVATIONS

3.1 Note on the Correlation of Observations Made by Dissecting Microscopy and Histology

A common frame of reference for observations on general morphology and histological structure was obtained by comparing the number of rows of primordia (and, in some cases, the number of primordia) visible under the dissecting microscope, with the number of definitive primordia (those with a recognisable placode and condensation) counted in serial transverse or tangential sections of the same specimen (Table 3.1). The same number (to within one row or one primordium) was detected by both methods. Neither method consistently revealed more primordia than the other. Where a row of primordia was just visible in the dissecting microscope, histology revealed the earliest stages of dermal condensation (described in Chapter 5).

Thus, row for row, descriptions of primordia in living and fixed material, from freshly excised or cultured skin, are directly comparable.

TABLE 3.1 Comparison of Primordia Observed in Culture and in Histological Sections*

Specimen	Number of Primordia			
	Living Specimens		Histological sections **	
	Rows	Individual Primordia	Rows	Individual Primordia
1	3		3	
2	6		6	
3	9		9	
4	4		4	
5	8		7	
6	6	20	6	20
7	6		6	
8	8		8	
9	6		7	
10	7	17	7	17
11	3		3	
12	6		5	
13	6		6	
14	5		5	
15	7		8	
16	5		4	
17	6		5	
18	3		3	
19	3		3	
20	5		4	
21	3		3	

* Data for some of these specimens is also included in Table 5.1.

** Specimens 1 to 11 - transverse sections; specimens 12 to 21 - tangential sections.

3.2 Observations on Living Material

Freshly Excised Skin

The following description is based on skins grouped according to the Hamburger-Hamilton stages of embryos from which they were excised. The incubation times are approximate.

Stage 29 (6 days 0 hrs.). No primordia are present. A thin, opaque line is visible posteriorly in the dorsal midline, and extends over two thirds of the length of the excised region. The skin is relatively opaque for about 300 μm laterally, on either side of this line.

Stage 29+ (6 days 6 hrs.). The medial line shows regularly spaced breaks at its posterior end, and in some - presumably older - specimens, along its entire length. The first row of primordia was beginning to form in most of the specimens examined: about four primordia lay on the midline near the posterior end of the specimen (Fig. 3.1a) each coincided - though not always precisely - with a dash on the medial line. At this stage, a majority of specimens (though not always those with a clearly defined row of primordia) showed low, medio-lateral ridges along their length: these are described in Chapter 5.

Stage 30 (6 days 12 hrs.). In most specimens 3 rows of primordia were present posteriorly. In the anterior one-third of the area excised, the medial row is absent or represented by one or two faintly visible primordia; the outer two rows diverge slightly. On either side of the mid-line, a strip of opaque tissue similar to that which previously lay adjacent to the mid-line now extends at least 200 μm beyond the rows of primordia, although its boundaries are indistinct. Low, medio-lateral ridges were present in a majority of specimens.

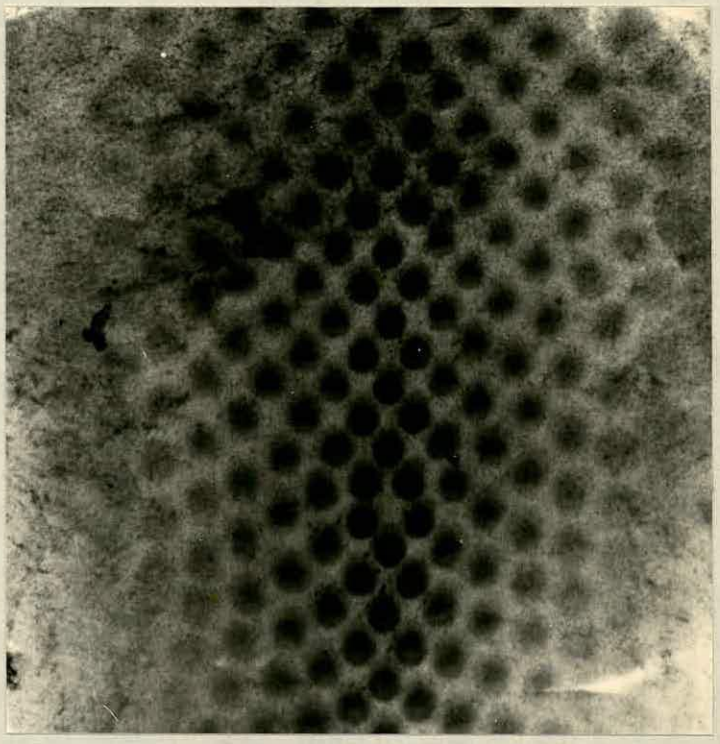


Fig. 3.1 Primordium pattern in freshly excised skin; Stage 33; 8 days development. Anterior is to the top of the picture.

Stages 31 and 32 (7 to 7½ days). Six to eleven rows of primordia are present. The most lateral primordia are only slightly more opaque than the surrounding tissue and are distinguished by a very slight modulation of the skin surface when the specimen is viewed obliquely: the optical density of primordia is greater medially. Primordia of the three or four most medial rows are optically dense and have a sharp outline; the skin between them is less opaque than elsewhere. Combined normal and oblique examination indicated that the optical density of primordia increases with their elevation above the skin surface, and that primordia with a sharp outline are at the bud stage. The feather pattern is extremely regular. One more row is present anteriorly than posteriorly, on either side of the pteryla. Low, medio-lateral ridges were present in some of the specimens examined. The thigh pteryla is beginning to form. The area of the excised region is about 30% greater than at Stage 29+.

Stage 33 and later (8 days and onward). About 16 rows of primordia are present at 8 days (Fig. 3.1), and the maximum of 17 or 18 rows is achieved shortly afterwards. Existing primordia continue to develop, being most advanced medially where they begin to form feather filaments. About half the specimens examined showed brown pigment granules which, though present throughout the skin, were especially concentrated in primordia. Pigmentation begins medially and spreads laterally. The area of the excised skin is about 50% greater than at Stage 29+.

Specimens Developing in Culture

The earliest stage at which skin can be explanted and its development maintained in culture is Stage 29 (5 days 20 hrs. to 6 days 0 hrs.). Younger specimens degenerate after two days without

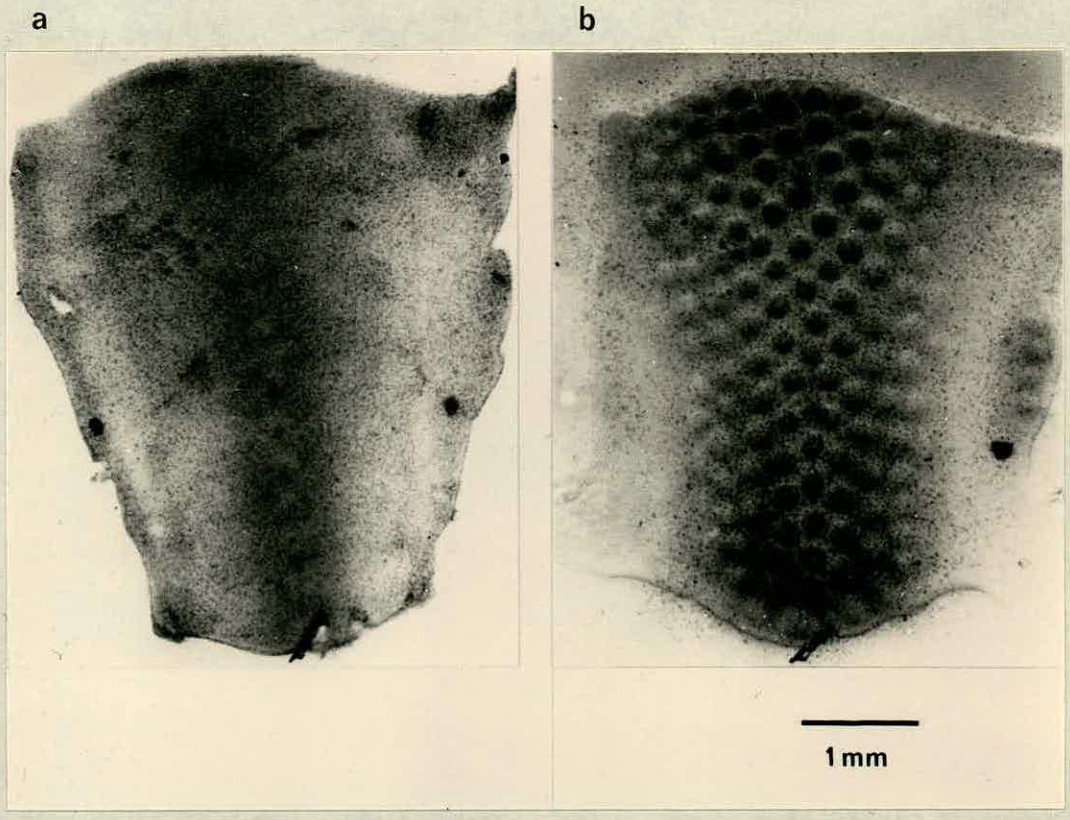


Fig. 3.2 Skin developing in culture.

a. Immediately after explantation at Stage 29+.
b. On the second day of culture; equivalent to Stage 32+.

Anterior is to the top of the picture.

forming primordia. Degeneration is accompanied by shrinking and atrophy of the epidermis and mass outgrowth of dermal cells over the substratum. In contrast, specimens explanted at 6 days 0 hrs. reliably develop.

Within the first three hours after explantation, the skin shrinks slightly: its linear dimensions decrease by about 10% both anterio-posteriorly and medio-laterally, though the degree of shrinkage varies between specimens. (Skin which is allowed to float freely in culture medium curls, dermis inward, within a few minutes after excision, suggesting that the dermis contracts more than the epidermis under these conditions.) After 8 to 12 hours in culture, the skin has become firmly anchored to the substratum and a few dermal cells can be seen moving out from its edges. Those specimens which showed mediolateral ridges at the time of explantation showed none after the first few hours in culture.

Once the development of primordia begins, the general impression is that it closely resembles development in vivo. Specimens explanted at Stage 29 pass through the same changes as were observed in successive stages of freshly excised skin: the first row of primordia forms 6 to 12 hours after explantation and the size of primordia and the spacing between them is approximately the same as in freshly excised skin.

Four points on which development in culture differs from development in vivo were noted (compare Figs. 3.1 and 3.2).

First, recently formed primordia (those in the 3 or 4 most lateral rows) appear, by normal and oblique examination to be slightly more elevated than those in equivalent freshly excised skin. This appears to be an immediate consequence of the excision procedure for when skin in which several rows were already present was

explanted, within three hours (concurrent with shrinkage) primordia younger than the feather-bud stage became slightly more elevated and assumed the same appearance as those in skin which had developed in culture. This change is not due to flattening on the substratum, for the same changes occur in freshly excised skin which is allowed to float freely in culture medium. In no case did primordia disappear or the pattern become re-organised in culture as described by Novel (1973).

Second, the period between the initiation of successive rows of primordia is longer in culture than in vivo (about 8 hrs. in culture compared with about 6 hrs. in vivo). (See Fig. 3.3). The rate of initiation of successive rows is approximately linear in culture, and does not depend measurably on whether the culture medium is changed daily or left unreplenished. The full compliment of 10 to 12 rows is formed about 50 hrs. after the first row appears.

Third, the feather pattern formed in culture, though remarkably consistent and similar to the pattern formed in vivo, shows local anomalies. For example, single primordia may be missing or displaced from the positions expected in the normal pattern; adjacent primordia may be fused; primordia may be larger or smaller than normal. The great majority of specimens examined each showed only one or two minor anomalies; generally, these included displacements and variations in the size of primordia and were more frequently found anteriorly than posteriorly. By far the largest number of anomalies were found in a few specimens which had been slightly distorted during explantation. The pattern formed in cultures is described and discussed in more detail in Chapter 4.

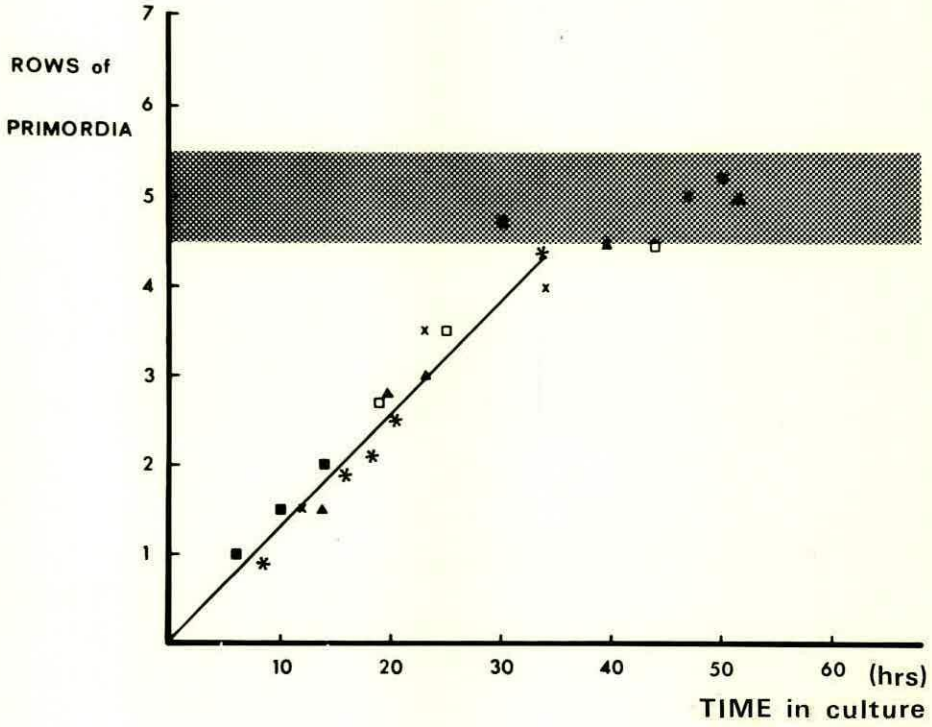


Fig. 3.3 Rate of initiation of primordia in cultured skin. y-axis shows number of rows on either side of, but excluding medial row. Each symbol represents a mean of rows counted in (n) specimens from one experiment. ■ (2); □ (3); Δ (2); x (2); ✻ (4). Time for each specimen is the time of formation of the initial row. ▒ region where the number of rows approaches the maximum for the width of the explant and where rows versus time relation is not, therefore, resolved within the interval between observations. Line: least squares regression on all values excluding these greater than 4.5. (See Table 3.2).

TABLE 3.2 Initiation of new rows of primordia versus time in culture.

Experiment	Mean Number of rows of primordia on either side of the midline excluding the medial row												
	C50			C132**		C201**				C203		C204	
Specimen	2	4	6	3	5	1	3	4	5	2	6	1	3
Time (hrs.) in culture*													
0	0	0	0	0	0	0	0	0	0	0	0	0	0
6												1	1
8.5						1.5	0.5	0.5	1				
10												1	2
12				1	2								
13.75										2	1		
14												3	2
16						2.5	1.5	1.5	2				
18.25						2.5	2.5	1.5	2				
19	3	3	2										
19.5										2.5	3		
20.25						3	3	2	2				
23				3	3.5					3	3		
25	4	3.5	3										
30												3	6.5
33.75						5	4.5	4	4				
34				4	4								
39.5										4.5	4.5		
44	5	4.5	4										
47				5	5								
50												4	6.5
51.5										4.5	5.5		

For graph of data, see Fig. 3.3.
Lease-squares regression line based on all counts excluding those at anytime where one count is $\geqslant 4.5$: $y = 0.126x + 0.054$.
correlation co-efficient = 0.864.

* Time after formation of medial row in each specimen.
** Medium on cultures was changed for fresh medium: C132, at 14 hrs.
C201, at 15 hrs.

Fourth, in striking contrast to skin developing *in vivo*, the area of the explant does not increase measurably during the first three days in culture. However, cell outgrowth does occur. All round the explant, individual dermal cells move out over the surface of the collagen and into its hydrated lattice. No pattern was observed, in these outgrowths, which could be related to cell patterns found in intact dermis. Locally, sheets of epithelial outgrowth colonise the surface of the substratum. Generally, during the first three to five days, cell outgrowth is not extensive and has no marked effect on the integrity of the skin: only exceptionally was excessive outgrowth observed. After 5 or 6 days in culture most specimens increased in area by spreading laterally: primordia became more widely spaced than before. This spreading did not, however, resemble the co-ordinated growth which occurs *in vivo*: it was associated with extensive and disordered outgrowth of cells from the explant margin and resulted in irregular distortion of the skin and of the feather pattern.

The later stages of skin development in culture also closely resemble development *in vivo*. Where parts of the thigh pteryla were incorporated in the explant, these began development on the second day of culture, approximately in keeping with their normal time schedule. Primordia of the spinal pteryla continue to develop through the feather bud and filament stages: filaments attain a length of about 1 mm. At this stage, however, morphogenesis is slow and, after a total of about seven to eight days in culture, development comes gradually to a halt. The integrity of the explant is maintained till 10 to 14 days after explantation, then the skin disintegrates in association with extensive cell outgrowth.

3.3 Observations on Fixed Material

Preliminary Examination of Histological Preparations of Filter-Mounted Skin: Comparison with Skin Sectioned *in situ*

Since descriptions in this and later chapters are based on observation of sections of filter-mounted skin, it is necessary to establish that this method of preparation does not unduly distort the structure of the skin. I therefore give a brief account of a comparison of freshly excised, filter-mounted skin with skin sectioned *in situ*.

Detailed examination of sections prepared by either method showed all the well-known features of developing skin. However, in examining cell patterns in the dermis tangential sections of mounted skin were found to be far superior to tangential sections of skin *in situ* for, in the former, extensive areas of pattern were visible in individual sections whereas, in the latter, a picture of cell orientation had to be pieced together from observations made on several sections in series. The histological structure of primordia in both types of preparation was virtually identical. Only two differences were noted: first, primordia of the two most lateral rows were generally slightly more elevated above the skin surface in mounted skin than *in situ*; second, cells of the subdermal mesenchyme were loosely arranged *in situ*, but were packed and aligned parallel to the filter surface in mounted specimens. However, in specimens mounted with the epidermis against the filter, cells of the subdermal mesenchyme were loosely packed and not aligned. (The same result was obtained when cultured skin was mounted epidermis to the filter.)

These observations are discussed in Section 4, but, for the present, it is clear that the structure observed in preparations of filter-mounted skin is closely representative of skin *in situ*.

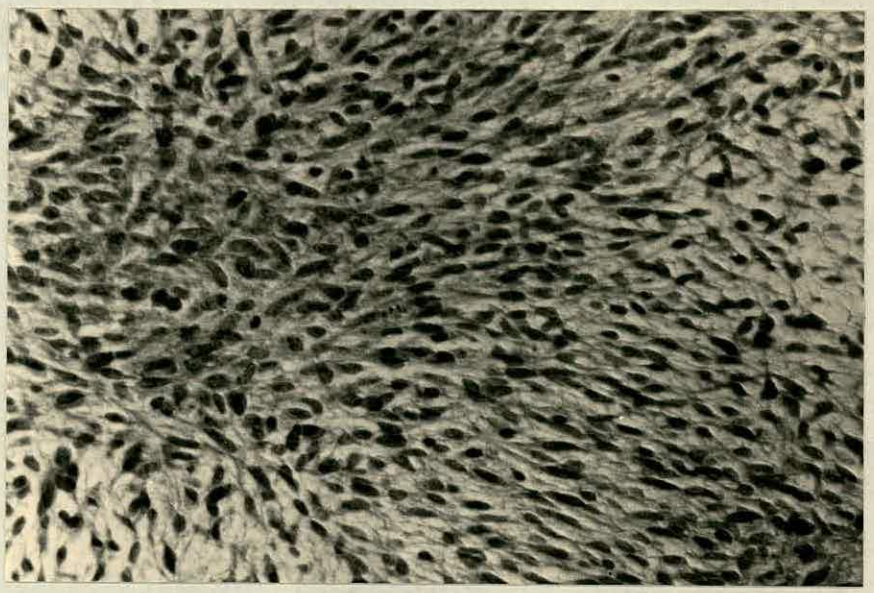
Skin Developed in Culture Compared with Freshly Excised Skin

The general structure of cultured skin closely resembles that of freshly excised skin (Fig. 3.4): primordia are clearly distinguishable with well developed placodes and condensations.

The following differences between cultured and freshly excised skin were noted.

Primordia of each row in culture appear to be more advanced in development than their counterparts in freshly excised skin. Three factors contibute to this appearance. First, placodes without associated condensations are rarely observed. Second, recently formed primordia are more elevated above the skin surface than those of equivalent rows in freshly excised skin. Third, cells are tightly packed in cultured condensations, even in the most lateral row: there is little or no unstained space between the cells and their nuclei are large, diffusely-stained and are approximately spherical. Cells are conspicuously arranged in a radial manner around each condensation, but few cells lie parallel to its circular boundary. The youngest condensations showing this arrangement of packing and orientation in vivo are those in the third- and fourth-most lateral rows. In contrast, cells in the two most recently formed rows in vivo are separated by conspicuous spaces and many are oriented paralled to the boundary of the condensation. (These features of in vivo development are described in Chapter 5; see, for example, Fig. 5.1.)

Most cultured specimens examined in tangential section showed clearly-defined intercondensation arrays, but these were never so well organized as arrays in vivo: nuclei were shorter and less uniformly oriented; cell processes were less straight, and possibly shorter, than in vivo. Cell density over the whole intercondensation



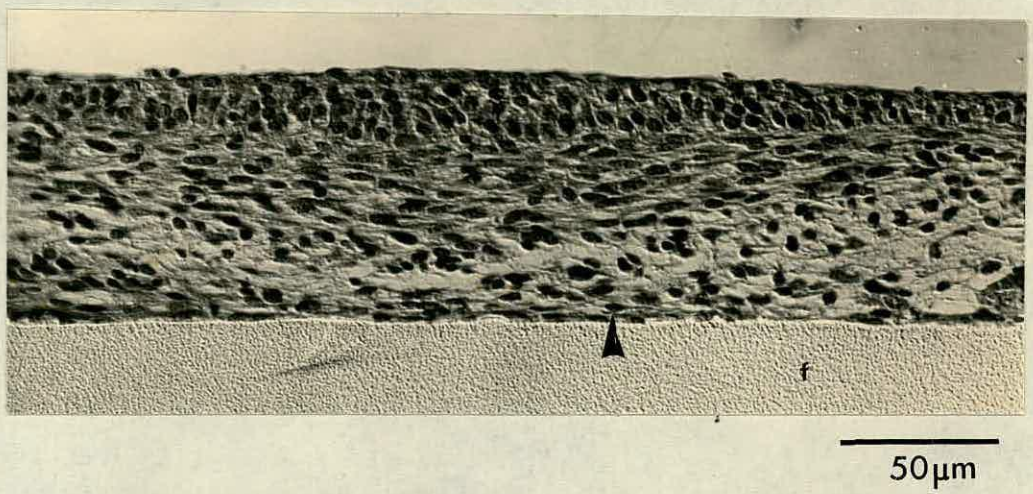


Fig. 3.4 Histological structure of dermis in freshly excised skin.

a. Condensation (left) and intercondensation array (right); tangential section.

b. Condensation; transverse section. Note aligned cells ► of the subdermal mesenchyme below the condensation. f = filter.

region appeared, by qualitative examination, to be greater in cultured than in freshly excised, skin; however, it is possible that this appearance was derived, in part, from the less well-organised structure of the dermis. In 11 cultured specimens, 3 showed no orientation of cells between well-formed condensations though, in some places, poorly organised arrays extended from recently-formed condensations towards presumptive sites of condensation. Experience in examining freshly excised skin indicates that cell orientation is less strikingly revealed in transverse, than in tangential, sections. Nevertheless, the majority of cultured specimens examined in transverse section showed few clearly oriented cells lying between condensations (Fig. 3.5).

In 6 of 11 specimens of cultured skin, a strip of dermis immediately lateral to, or co-incident with, the most recently formed row of condensations showed a proportion of cells oriented anterio-posteriorly. These cells were packed together and were shorter and their alignment was less uniform by comparison with cells in inter-condensation arrays. The degree to which this band was organised varied between specimens, from very marked in two specimens to barely discernable in two of the remainder. In freshly excised skin, anterio-posterior orientation was confined to small groups of cells which were no more common than groups oriented in other directions,

In all specimens of cultured skin examined in transverse section, the dermal-epidermal interface was smooth: no well-developed epidermal spurs were observed. However, fibres resembling anchor-filament bundles lay perpendicular to the dermal surface, and some of these were associated with very small, spur-like epidermal protrusions.

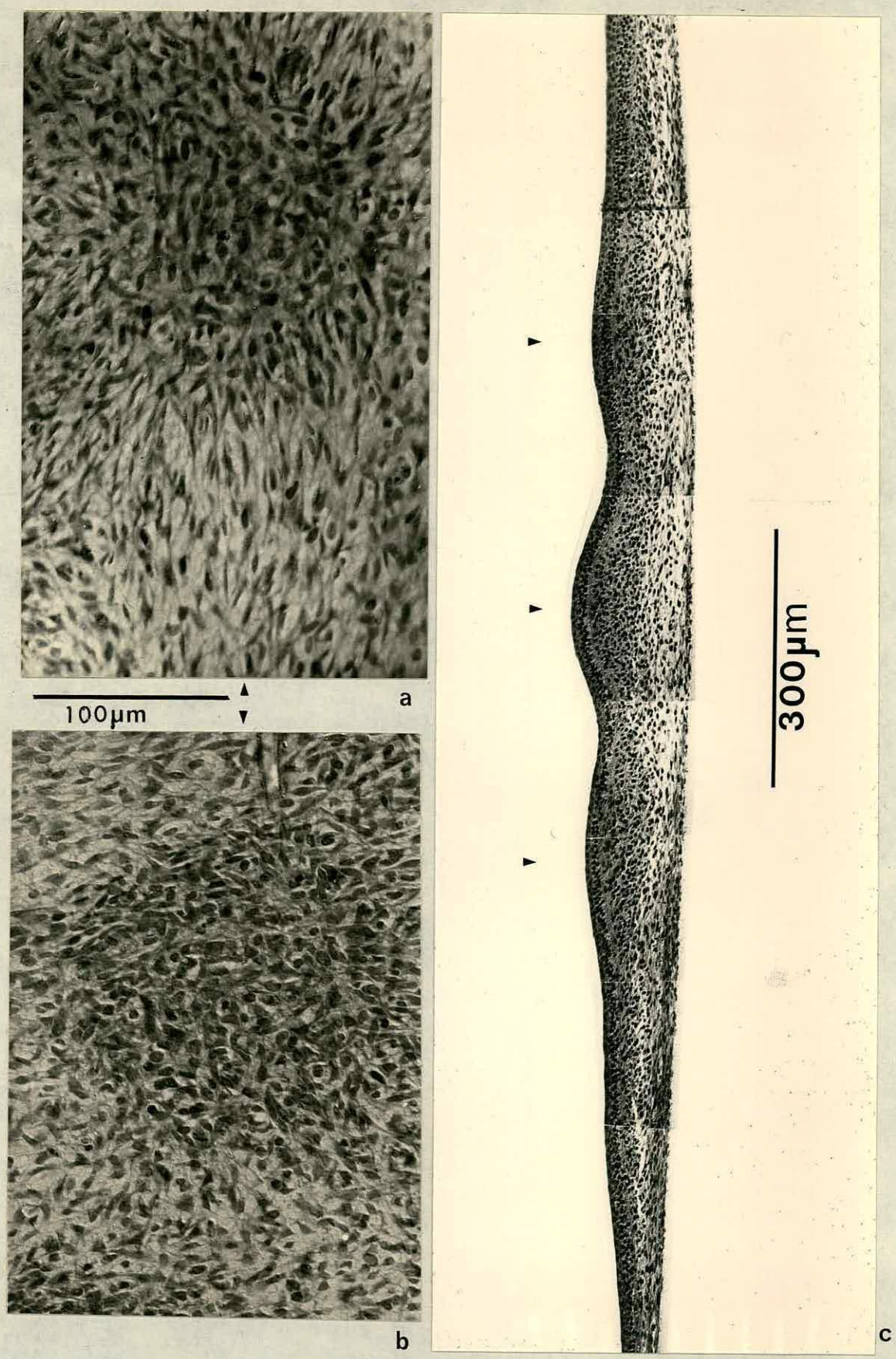


Fig. 3.5 Histological structure of dermis in cultured skin.

a. Condensation (top) and intercondensation array (bottom); tangential section.
b. Condensation; tangential section.
c. General view of skin; transverse section, arrows indicate primordia.

All of these anomalies are apparently products of development in culture, rather than immediate effects of explantation: 8 specimens which had been explanted when several rows of primordia were already present, and fixed after between one and six hours in culture had a structure indistinguishable from that of freshly excised skin.

4. DISCUSSION

The present culture method was devised to overcome difficulties inherent in other methods of culturing the skin. Culture on the CAM, while permitting successful development, has severe disadvantages for experimental studies: many kinds of experimental treatment are difficult to apply, continuous observation of the explant is not possible, and interpretation of the results is, in some cases, confused by possible effects of host embryos. To overcome these problems a variety of *in vitro* systems have been used for skin culture (Shaffer, 1956; Sengel, 1958; Bell, 1964; Wessells, 1965; Novel, 1973). However, in these methods the explant is supported on opaque or translucent materials, for example, agar or millipore filter, or is stretched between individual fibres of a fabric (Shaffer, 1956) which may, by stress or orientation of cells along fabric fibres, destroy the delicate organisation of the dermis (Mees, unpublished observation, quoted by Ede *et al*, 1971). Transparent collagen substrata have been used previously for the culture of cells (Ehrmann and Gey, 1956; Elsdale and Bard, 1972), but not to my knowledge, as substrata for organ culture. In preliminary trials using several different methods the present system was

found to be superior in terms of ease and reliability in setting-up the culture, in applying experimental treatments, and particularly in observing the development of the explant continuously under conditions yielding good optical resolution. No detailed comparison has been reported, or made here, between development in other culture systems and development *in vivo*. However, there is no reason to suppose that most of the anomalies observed in the present system are particular to the collagen method, rather than a reflection of the general effects of excision and *in vitro* culture.

The main purpose of the observations described here is to characterise skin development in the present culture system. By showing the similarity of development in culture and *in vivo*, the observations described here point to the potential of this method as a useful tool for analysing the problems posed in Chapter 1. The system may also prove useful for culturing other organs. Skin explanted before any morphological signs of primordia are present will form primordia which develop into filaments 1 mm long. The pattern of development and the general appearance and several aspects of the histological structure of cultured skin resemble skin developing *in vivo*. No biochemical comparison has been made so that the similarity of molecular aspects of development *in vivo* and in culture cannot be judged.

In addition, the results indicate the usefulness of the ancillary methods for observing skin structure. Dissection microscopy of freshly excised, or cultured, skin mounted on collagen gives a clear and detailed view of its general morphology which can be directly correlated with its histological structure. Histology of filter-mounted skin, which is especially useful in revealing dermal

cell patterns, causes only slight distortions in tissue structure, as defined by reference to skin sectioned in situ. One of these distortions - the packing of subdermal cells - is apparently a simple consequence of the collapse of weakly-structured sub-dermal mesenchyme against the filter, for it is not found in skin mounted epidermis to the filter. The second distortion - the elevation of young primordia - is discussed below.

The present observations do, however, point to some important anomalies in the development of cultured skin. Clearly, these must be considered in interpreting observations and experiments. In addition, they themselves provide insight into the mechanisms of normal development. These points are dealt with as they arise in later chapters. Here, discussion is limited to a general consideration of the effects of excision and culture.

Anomalies in General Development and Morphology

The earliest observed effect of excision and explantation is a slight, but quite rapid, shrinking of the skin and, in particular, of the dermis. Novel (1973) similarly found that the skin shrank more or less severely within 1 or 2 hrs. of explantation. Abercrombie (1970) briefly reviews evidence that meshworks of fibroblastic cells which lack attachment to a solid substratum have an internal tension and suggests that this is related to the contractile response of cells to mutual contact. Whatever the cause of shrinkage at a cellular level, it clearly indicates that a new distribution of forces becomes operative in the excised skin and it seems probable that these forces are also responsible for the concurrent, slight elevation of young primordia which occurs when skin with several rows already present is explanted. The same forces may contribute to the elevation of young primordia developing

in culture.

These effects of explantation on cultured material must be distinguished from shrinkage which may result from fixation and histological processing, and which may also contribute to the increased elevation of young primordia in histological preparations of mounted skin, whether freshly excised or cultured, as compared with skin fixed and processed in situ. Sengel and Mauger (1976) similarly noted that primordia become more elevated after fixation in gluteraldehyde and osmium tetroxide and processing for SEM: they attributed this, in part, to shrinkage. A second factor which may contribute to the elevation of primordia in mounted material is the fact that even partly developed condensations protrude into the subdermal mesenchyme in situ. Since this tissue collapses on mounting, condensations - and thus whole primordia - will be pushed upwards by the filter.

Skin becomes able to develop in the present culture system at the same well-defined stage (Stage 29; around 5 days 20 hrs.) as was reported by Bell (1964) and Wessells (1965) using quite different culture conditions. Bell (1964) has suggested that, before this stage, skin development requires humeral factors from the living embryo. The observation of the same apparent threshold under a wide range of culture conditions lends circumstantial support to this hypothesis, but, in the absence of positive evidence or a critical test of this idea, the reason for this apparent threshold must remain uncertain.

Once the development of primordia has begun, it is clear that successive rows take longer to form in culture than in vivo: 12 rows form in about 50 hours in culture, while 17 rows form in the same time in vivo. The most obvious explanation of this is that a

particular or general physiological deficiency of the culture situation limits the rate of the processes involved in the initiation of new rows. However, three observations cast some doubt on this interpretation. First, the rate of initiation might be expected to decrease over the first two days in culture as any crucial substances which might be present in the skin or in the medium are depleted, but the rate is approximately linear. Second, changing the culture medium, which might be expected to change the rate of initiation, had no observed effect. Third, rates showing close approximation to the same curve were observed in experiments conducted as much as two years apart, using different batches of medium, serum, etc. An alternative explanation will be considered in Chapter 6 in the light of later results. It is clear that the relatively long initiation period could account, in part, for the older appearance of primordia formed in culture as compared, row for row, with primordia in vivo, if the initiation rate is slowed, while subsequent development is not.

The lack of coordinated skin growth in culture, which has been noted by several workers, and also applies to skin growing on the CAM (Linsenmayer, 1972), may be caused by several factors, for example, a change in the distribution of physical forces, or a lack of specific physiological factors. However, there is no evidence to favour one or other of the possibilities. Given that the diameter of primordia is approximately fixed, the lack of normal growth accounts for the fact that only 10 to 12 primordia fill the width of the pteryla in culture, whereas 17 or 18 rows form in vivo. In addition, the fact that the behaviour of cells at the edge of the explant resembles that of cells growing singly in culture points to a lack of integration within and between the tissues at the explant margin. This phenomenon is, of course, common to most organ culture

situations, though the nature of the tissue organisation which is so obviously lost is not known.

Finally, it is not surprising that the period in which skin will develop in culture has a well-defined end-point, just as it has a well-defined beginning: the lack of a blood supply alone would be expected to halt filament development (Goff, 1949).

Anomalies in Structural Development

The most important structural differences between cultured and freshly excised skin are in the organisation of condensations and of the cellular arrays between them. Although the initial structure found in condensations in vivo appears to exist for only a very short period, or may even be by-passed altogether in culture, it seems very unlikely that condensations develop in an essentially different way from those formed in vivo since the eventual structure formed is similar to that found in more advanced stages in freshly excised skin. The study of the condensation process in culture (Chapter 5) is based on this assumption. It is clear, however, that some aspects of this process differ in vivo and in culture. In particular, the relation of the condensation process to the orientation of intercondensation cells may be changed, although it is clear from the present observations that condensations can form in association with relatively disorganised dermal cell patterns. The relative timing, and proportional contribution of different factors to the process will also be expected to vary in culture from those found in vivo, so that it is not possible to extrapolate directly from studies in culture to the more subtle aspects of the process in vivo. The possible causes of these structural anomalies will be discussed in Chapter 5 in the light of a morphological description of the

of the structures formed *in vivo* and an experimental analysis of the process in culture.

It is not clear what causes the predominant anterio-posterior orientation of cells at the medial boundary of the dense dermis in some specimens of cultured skin, but this may be the result of lateral compression forces which could arise through lack of marginal growth combined with continued medial development of dermal condensations. The morphology of the short, often closely packed cells in this area supports the possibility that these alignments arise through cell packing rather than stretching. This cell arrangement does not grossly affect the regular nature of the pattern formed in culture.

Finally, the presence of anchor filaments below the smooth epidermal-dermal interface indicates that filaments can form in the absence of epidermal spurs, although the reason why the interface is smooth is not clear.

CHAPTER 4

THE TIME AND POSITION OF PRIMORDIUM MORPHOGENESIS

1. INTRODUCTION

The first step in an investigation of the relation between dermal morphogenesis and the time and position of primordium development is to describe the formation of the pattern *in vivo* in morphological and histological terms. Published accounts cover some of this ground thoroughly, but they are incomplete or conflicting on certain crucial points. These points are examined in the first part of the present chapter. In the second part of the chapter, the pattern formed in culture is described, and investigated experimentally.

2. OBSERVATIONS ON FRESHLY EXCISED SKIN

2.1 General Morphology of the Emerging Feather Pattern

Although accounts of the feather pattern at 10 to 12 days development have been published (reviewed in Chapter 1), whether the chevrons are curved or straight as the pattern emerges has not been established.

Twenty-eight freshly excised specimens, which had 7 or more rows of primordia, were examined, mounted on flat collagen substrata or on glass coverslips. Twenty-one of these showed curved chevrons in a pattern similar to that described in published accounts of 10 to 12 day-old embryos (see, for example, Fig. 3.1). In the remaining specimens chevrons were straight or nearly so. In the anterior part of the excised region, primordia were slightly more widely spaced and less regularly arranged than in the posterior part.

In several of these specimens very faint medio-lateral stripes were observed in the lateral, undeveloped parts of the pteryla. These corresponded in number to the primordia in the initial row. Oblique examination showed that these were broad low ridges, but their structure has yet to be investigated.

2.2 Extent of Dermal Cell Patterns Lateral to Existing Primordia

Previous histological accounts have given conflicting reports of the extent of cell patterns in the dermis lateral to existing primordia (reviewed in Chapter 1). This is most likely due to limitations of the methods used to examine the skin. Whole mounts of dermis (Stuart et al, 1967, 1972; Ede et al, 1971) give poor resolution. To cut histological sections across the back of the chick (Ede et al, 1971) also has disadvantages: only small areas of the section pass parallel to the curved surface of the skin, and there is a danger that well-formed condensations or arrays cut obliquely are mistaken for very early condensations or arrays passing into lateral dermis. The only histological studies of skin cut strictly parallel to its surface, after removal and mounting on a flat surface, are those of Wessells and Evans (1968) and Sengel and Rusaouën (1969). The former authors found that only scattered fibroblasts are oriented towards points where future primordia will form. The latter report dealt only briefly with dermal structure lateral to formed primordia. However, Sengel later noted that arrays converge on presumptive sites of new condensations (Sengel, 1971, 1976b), and suggested (in a communication noted at the end of the report by Wessells and Evans) that the discrepancies between accounts may be due to sampling at different levels below the dermal surface. To resolve these problems in the present study, sections

were cut strictly parallel to the skin surface and the complete series examined through the dermis of each specimen.

Freshly excised skin was briefly examined in the dissecting microscope and the number of rows counted. Specimens were mounted and processed histologically. (16 specimens in all: 8 sectioned tangentially, 2 with 1 row of primordia, 6 with 5 to 7 rows; 8 sectioned transversely, with between 1 and 9 rows.) Specimens which were sectioned tangentially were selected from a larger study on the basis of technical quality; in particular, tangential sections were required to pass strictly parallel to the skin surface as judged while cutting the skin, and by the criteria described below.

The tangential sections of each specimen used in this study contained at least 4 condensations sectioned at approximately the same level in each anterio-posterior row. Each section showed an approximately symmetrical medio-lateral distribution of rows of condensations and lateral unpatterned dermis. Single sections about 18 to 24 μm below the dermal surface showed the same number of rows (to within one row) as were counted in the living state. These observations are taken to confirm that the sections passed effectively parallel to the skin surface.

Definitive primordia are taken as those in which condensations are clearly discernable by the structure described in Chapter 5 (illustrated in Fig. 5.1c). Sites where small groups of cells may be in the initial stages of condensation are not included in this definition.

In tangential sections, 3 (2 of which had only 1 row of primordia) specimens showed no clearly defined arrays lateral to the most recently formed definitive condensations: cells were arranged

radially round these condensations, and the presumptive sites of new condensations were occupied by apparently unpatterned dermis. However, in a majority of specimens (5), cells were organised into detectable arrays extending diagonally from neighbouring condensations in the most lateral row towards presumptive sites of condensation. In most cases, only a few strands of aligned cells were seen to extend to a point of intersection in the lateral dermis. In a minority of cases (2), well defined, though not fully developed, arrays were observed to intersect at presumptive sites of condensation or where condensations were just beginning to form (Fig. 4.1) and, in some areas, strands of aligned cells extended up to 100 μm beyond these intersections. In none of the specimens examined did aligned cells extend far enough laterally to intersect more than one row beyond definitive condensations. No patterns of cells or fibres were detectable in dermis lateral to arrays (Fig. 4.1c).

Observations made on transverse sections were in agreement with the findings of Sengel and Rusaouën (1969): for example, of 4 specimens at the three-row stage, 3 showed a single row of placodes lateral to the most recently formed definitive primordia on

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Fig. 4.1 (see over) The intersection of arrays of dermal cells at the site of an initiating condensation. Tangential sections through the dermis of Stage 31 skin. a. - low magnification view; lateral to the right. Extreme right, unpatterned dense dermis; right of centre, initiating condensation at the intersection of arrays which extend from two clearly formed condensations (left of centre, top and bottom). b. - high magnification view of the intersection of arrays in the same field. Note there is little or no sign of condensation development. c. - high magnification view of the dermis lateral to the intersection. Notice the absence of patterns of cell orientation.

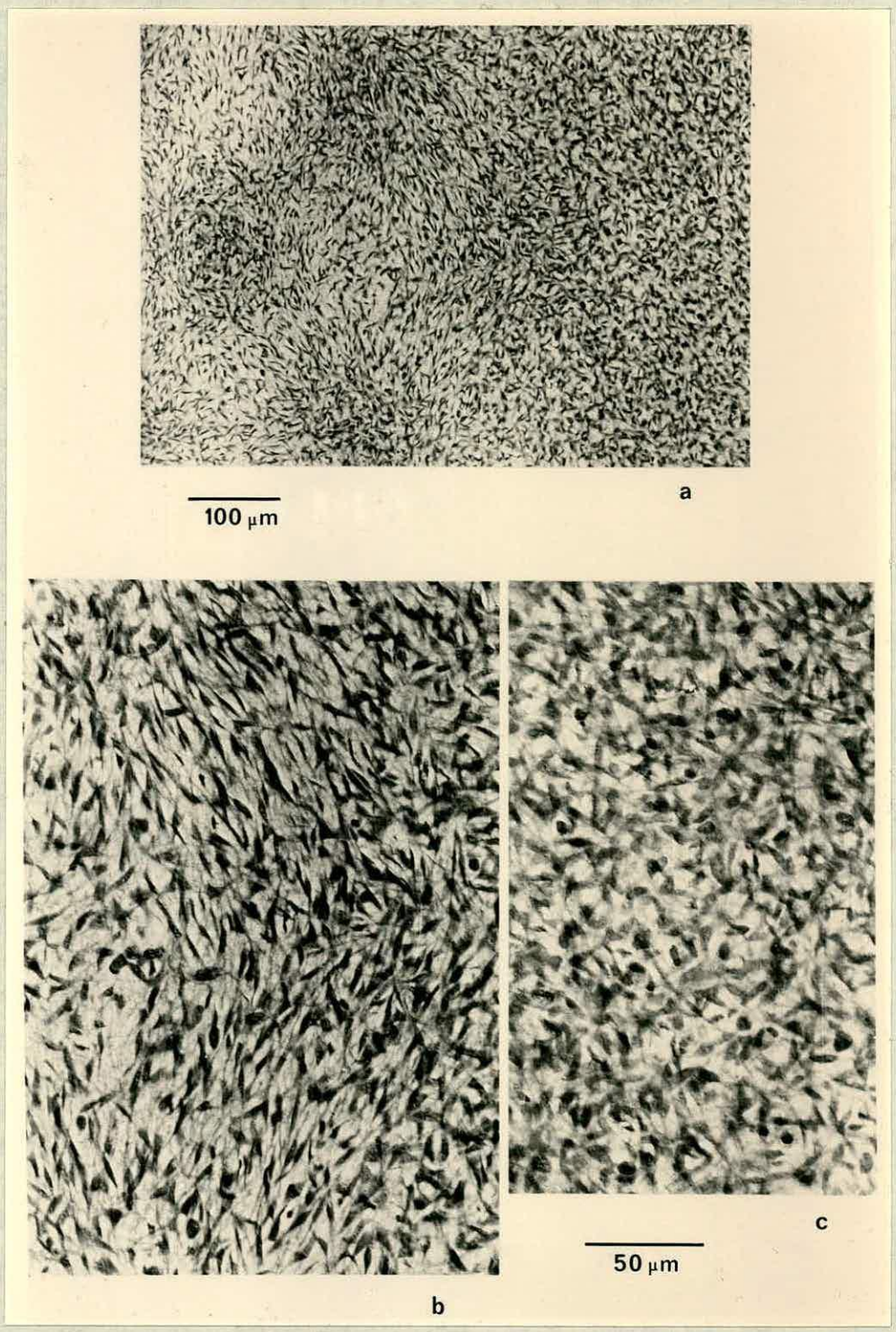


Fig. 4.1

either side of the pteryla, while the remaining case showed no unassociated placodes. The most lateral placodes were clearly distinguishable by their thickness (16 to 19 μm) from the surrounding dermis (12 μm thick laterally, but only about 6 μm thick medially). Dermal cells in approximately medio-lateral orientation - which presumably represented arrays - were observed to extend to beneath unassociated placodes and, in a few areas, to 100 μm beyond these. Lateral to this, no patterns were detectable in the dermis.

3. OBSERVATIONS ON CULTURED SKIN

3.1 General Morphology of the Pattern

The emerging feather pattern can be more closely followed in skin developing in culture than in freshly excised specimens. Moreover, several anomalies which develop in culture give additional insight into the pattern-forming process.

The following account is based on examination of 56 specimens developing in culture after explantation at Stage 29 to 29+. Additional observations, which were made while examining several hundred specimens in the course of the project, are noted with reference to specific points.

The pattern of the eight most posterior chevrons in the region examined was remarkably regular, with only occasional displacements or dual primordia in a few specimens (see, for example, Fig. 3.2). The most interesting feature of this region is the regular arrangement of chevrons; in forty-one specimens these were clearly curved, in fifteen specimens they were straight or nearly so. The curvature of chevrons was not due to distortion of the skin on

explantation, or to development or observation on curved substrata. Each specimen was floated onto the substratum taking particular care to minimise distortion. In each case, oblique examination established that the substratum was flat and that the specimen was only slightly thicker medially than laterally. In some cases, skin was removed from culture and carefully floated onto a plane glass cover-slip for final examination: the same curves were observed. Moreover, the symmetrical chevron curves in the posterior part of the pteryla consistently followed a pattern similar to that observed in freshly excised skin at Stages 31 to 33, and to the pattern reported in skin from 10 day old embryos (Sengel, 1976b) and in adult skin (Lucas and Stettenheim, 1972). The chevron pattern remained approximately constant for 1 to 2 days from the time lateral primordia were first visible.

With regard to the positions of individual newly formed primordia and the question of whether they lay equidistant from two neighbours in the preceeding row or lay strictly on a chevron curve the situation is complicated by several factors. First, trial measurements at high magnification of the distances between primordia did not prove useful because of the slight irregularity of the pattern and the small differences involved in distinguishing an equidistant position from one lying on a shallow curve. Second, in a few cases, the anterio-posterior rows on either side of the midline diverged slightly from a point just anterior to the centre of the explant so that, although new primordia lay on a curved chevron, they were approximately equidistant from two neighbours in the preceeding row. Third, in many cases, primordia were regularly spaced between one primordium in the preceeding row and two in the same row. Despite the variety of spatial relations between newly formed and

existing primordia, however, in the great majority of cases each new primordium lay more or less precisely on the extension of an existing chevron curve (Fig. 4.2) and, in a few cases, primordia lay on chevron curves, but were clearly not equidistant from their neighbours.

In the anterior region of the explant the pattern was regular only in a minority of specimens: in a majority of specimens primordia did not fall on clear chevron lines, but were more widely spaced and less regularly arranged than posteriorly (see, for example, Fig. 3.2) and the pattern was generally much less regular than that observed in vivo. The pattern showed differences between specimens; however, detailed examination suggested that all shared a common pattern from which variation arose through slight displacement or omission of a few primordia, through the presence of dual primordia, or, more rarely, through the addition of a single primordium. This common pattern was not however simply related to the in vivo pattern.

Displacements of several primordia in the same specimen appeared to be correlated through the effect of a single displacement on space available for primordia in neighbouring positions. For example, displacement of one primordium was frequently associated with displacement, reduction in size, or omission of surrounding primordia.

In most cases it was clear that dual primordia comprised two primordia which had individual places in the pattern and which were fused: no clear cases of duplication at a single position in the general pattern were observed. Fusions were observed between adjacent primordia in either the same, or succeeding rows (Fig.4.3). All degrees of fusion were found and in each case the anomaly could be observed from the earliest stage of primordium morphogenesis.

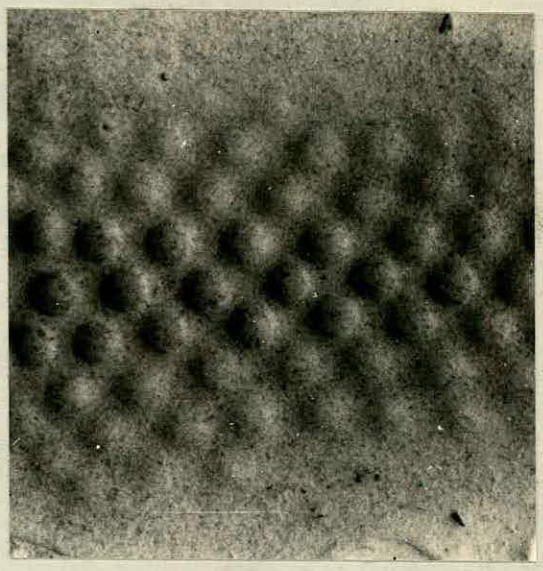


Fig. 4.2 Curved chevrons in skin explanted at Stage 29 on flat collagen, photographed on the second day of culture. Left, anterior.

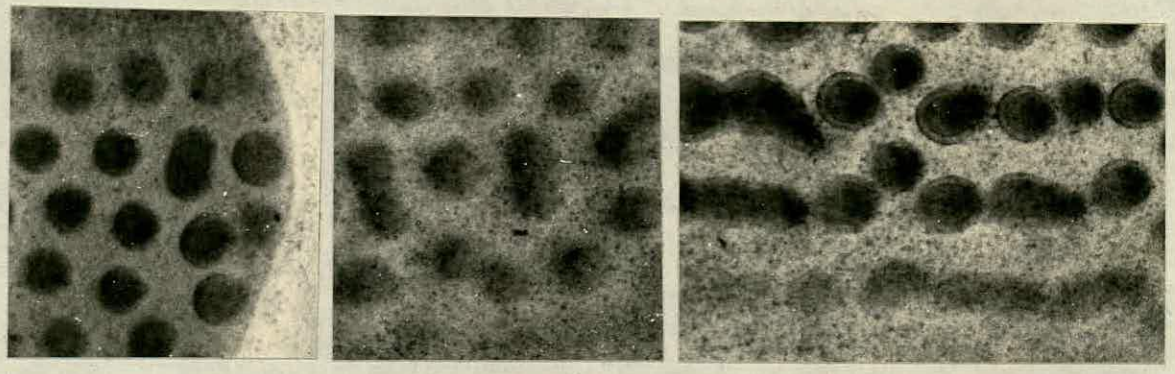


Fig. 4.3 Dual primordia formed in cultured skin.



Fig. 4.4 Cervical skin explanted at Stage 29+, photographed on the second day of culture. Right, anterior.

Some areas of pattern appear to be more fusion-prone than others: for example, dual primordia were much more commonly found in the anterior than in the posterior part of the explant and were most frequently found adjacent to the midline just anterior to the eighth initial primordium (counting from the posterior end of the excised region), approximately where the initial medial row terminated anteriorly in these specimens. In some specimens, the presence of dual primordia appeared to be correlated with restriction of the available space. In specimens examined in the course of other parts of this work, dual primordia occurred where the pattern was locally compressed, apparently in response to skin distortion (as, for example, in some cut specimens). In a single specimen, out of several hundred examined, all the primordia in each anterio-posterior row were fused.

In contrast to the irregular anterior patterns formed in specimens explanted at Stage 29 to 29+, specimens which were explanted when three or more rows were present formed a regular pattern anteriorly, with clearly defined chevrons. Finally, in 5 explants of the anterior and posterior cervical region from Stage 29 embryos, the same general patterns were observed from the earliest stages of morphogenesis (including the size of primordia, the direction and curvature of the chevrons, Fig. 4.4) as were observed in 7 freshly excised control skins from this region, and as described in published accounts of pteryla in 10 to 12 day-old embryos (Sengel, 1975).

3.2 Experiments on Pattern-Formation in Culture

The results described in Section 2.2 suggest that the dermis is morphologically unpatterned over almost the entire width of

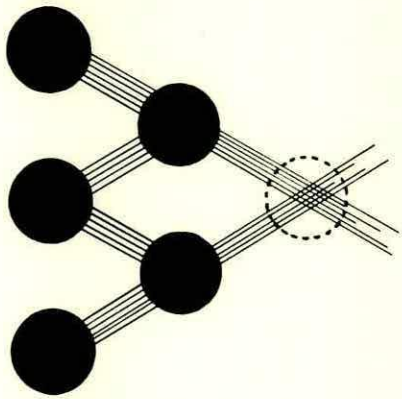
the pteryla at Stages 29 to 29+, except for a zone not more than the equivalent of about one row lateral to the midline. This opens up the possibility of investigating the rôle of the lateral extension of dermal arrays from existing primordia in determining the feather pattern.

The Patterning Rôle of Dermal Arrays

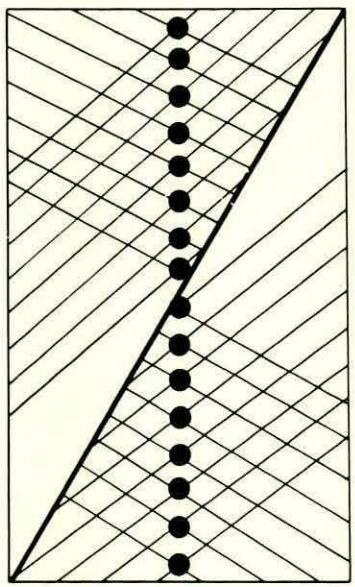
The model of Stuart et al (1972) (Fig. 4.5a) is open to a simple, but crucial, test. The model predicts that when skin at Stage 29 to 29+ is cut diagonally, specific large areas in the lateral part of the pteryla will remain unpatterned (Fig. 4.5b).

Skin was explanted at Stage 29 to 29+, then immediately cut with scissors (Fig. 4.6a). Its development was followed for two to three days in culture. (4 independent experiments; in all, 5 specimens cut diagonally with the anterior end of the cut on the right side, 6 specimens cut diagonally in the opposite way, 8 uncut control specimens.)

All but a single control developed normally; this one control failed to develop beyond the three-row stage. In all but three cut specimens, primordia developed in the usual pattern over the entire pteryla on both sides of the cut. The pattern developed in the normal sequence: primordia in each anterio-posterior row developed concurrently. Individual primordia formed close to the cut, though, in some cases, tissues at the cut margin extended slightly beyond these primordia during the remainder of the culture period. (Phase contrast examination of the cut showed no clear patterns of cell migration which might suggest the continued extension of dermal arrays over the substratum.) Primordia close to the cut lay in precise register with the rest of the pattern. The same number of rows of primordia was formed in both pieces of the



a



b

Fig. 4.5 Diagram to show model of Stuart et al and a critical experiment to test the model.
a. The model. ● formed primordium, ≡ arrays of dermal cells. The arrays intersect at the site of a new primordium ○ .
b. Test. Circles represent the initial row in spinal pteryla, heavy line represents a diagonal cut, light lines represent dermal arrays extended in a diagonal lattice from initial primordia. The cut interrupts arrays leaving large, specific areas of skin with no patterned lattice. According to the model, primordia will form only at intersections of the lattice, leaving large, unpatterned or bare areas.

same specimen and the rows corresponded approximately in position (though this could not be judged precisely).

In one of the remaining three cut pieces (Fig. 4.6b) development on one side of the cut followed the above description. The pattern on the other side of the cut was distorted as if it had been stretched. Despite this, primordia developed over the whole area of the pteryla and the general nature of the pattern was regular.

In two cases development ceased where only 5 rows had formed. These 2 specimens were from the same experiment and one of the 2 controls in this experiment failed to develop normally (see above). The rows which did develop on either side of the cut formed as described above.

Thus, in all, 17 out of 22 diagonally cut sides formed regular, normal patterns over the entire pteryla.

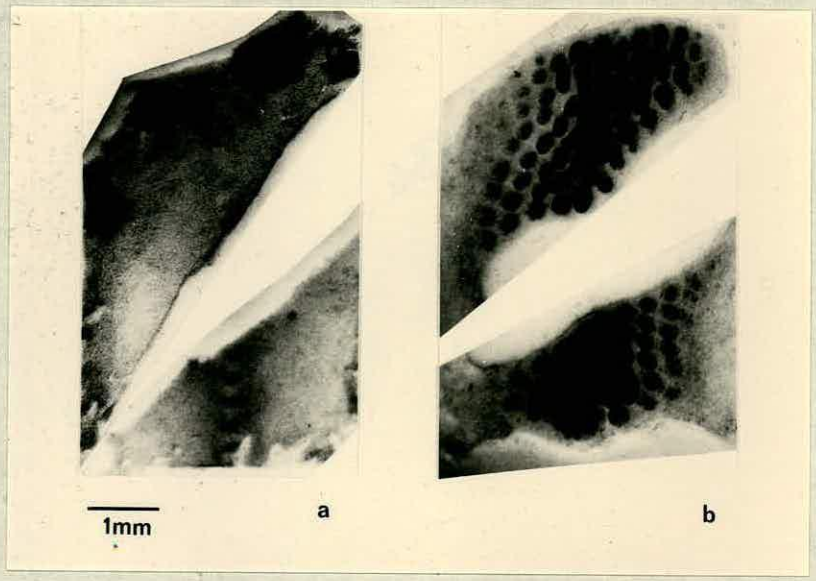


Fig. 4.6 Specimen developing in culture after cutting diagonally at Stage 29+. Note the regular feather pattern in the lower piece (posterior), and the distortion of the pattern in the upper piece. a. immediately after cutting; b. after 2 days in culture.

Does Primordium Formation Depend on the Propagation of an Activated State?

A more general investigation of the organisation responsible for initiating primordia was made by testing the hypothesis that an activated state, or a 'wave of morphogenesis' (Sengel, 1976b), is propagated through the pteryla immediately in advance of primordium morphogenesis.

The approach utilised the same type of experiment as used by Linsenmayer (1972) to investigate the importance of the initial row in the development of the thigh pteryla: that is, a piece of the presumptive pteryla was severed at an early stage from the region containing the presumptive site of the initial row, and its development followed. However, as Linsenmayer has emphasised, it is crucial to observe the time at which the first row is formed beyond the cut relative to the time at which a row is formed in an equivalent position in uncut skin. This provides an indication of whether the first row forms under the normal impulse or arises by a different mechanism, for example, as a regulative response to cutting. The present culture system allowed direct observation of the time of formation of new rows. Making use of the bilateral symmetry of the spinal pteryla, direct comparison could be made between cut and uncut sides of the same specimen.

Specimens were cut immediately after explantation at Stage 29 to 29+. In some cases, freshly excised specimens were placed on a glass cover-slip and cut with a scalpel, then transferred to a culture dish: this method allowed precise location of the cut. In other cases, specimens were explanted in a culture dish and cut with scissors. No differences were observed in the development of specimens cut by these two methods.

In the simplest cases, cuts were made unilaterally, parallel to the midline and at different distances from it. In some of these cases, the cut was made lateral to the lateral boundary of the relatively opaque, medial tissue (see description of skin in Chapter 3, Section 3.2). In two cases, severed pieces of the same specimen were cultured in separate dishes. A variety of other cuts were made to test specific points (treatments to each specimen are shown in Fig. 4.7). Development was followed for 2 to 10 days in culture. In most cases, specimens were observed at 2 to 3 hour intervals over the time when primordia were forming adjacent to the cut. In all, 5 independent experiments were carried out, including 13 cut specimens and 10 uncut controls (each specimen is listed in Table 4.1 and Fig. 4.7).

All control explants developed normally.

In all cut specimens, except where the cut was made at the distance equivalent to 1 to 2 rows from the lateral edge of the pteryla, primordia developed in pieces of the pteryla severed from the site of the initial row: primordia developed normally over the remainder of the pteryla (Fig. 4.8). Depending on the distance of the cut from the midline, between 1 and 3 rows of primordia developed medial to the cut (excluding the initial row). The same result was obtained when pieces of the pteryla were cultured separately. In all but two cases, the feather pattern showed normal regularity. In the remaining cases, the pattern was distorted as if the skin had been locally stretched: anterio-posterior rows were curved, and several dual primordia were observed. In 2 cases, although lateral severed pieces developed primordia, fewer rows formed over part of the area than over the equivalent region on the uncut side (see, for example, Fig. 4.8).

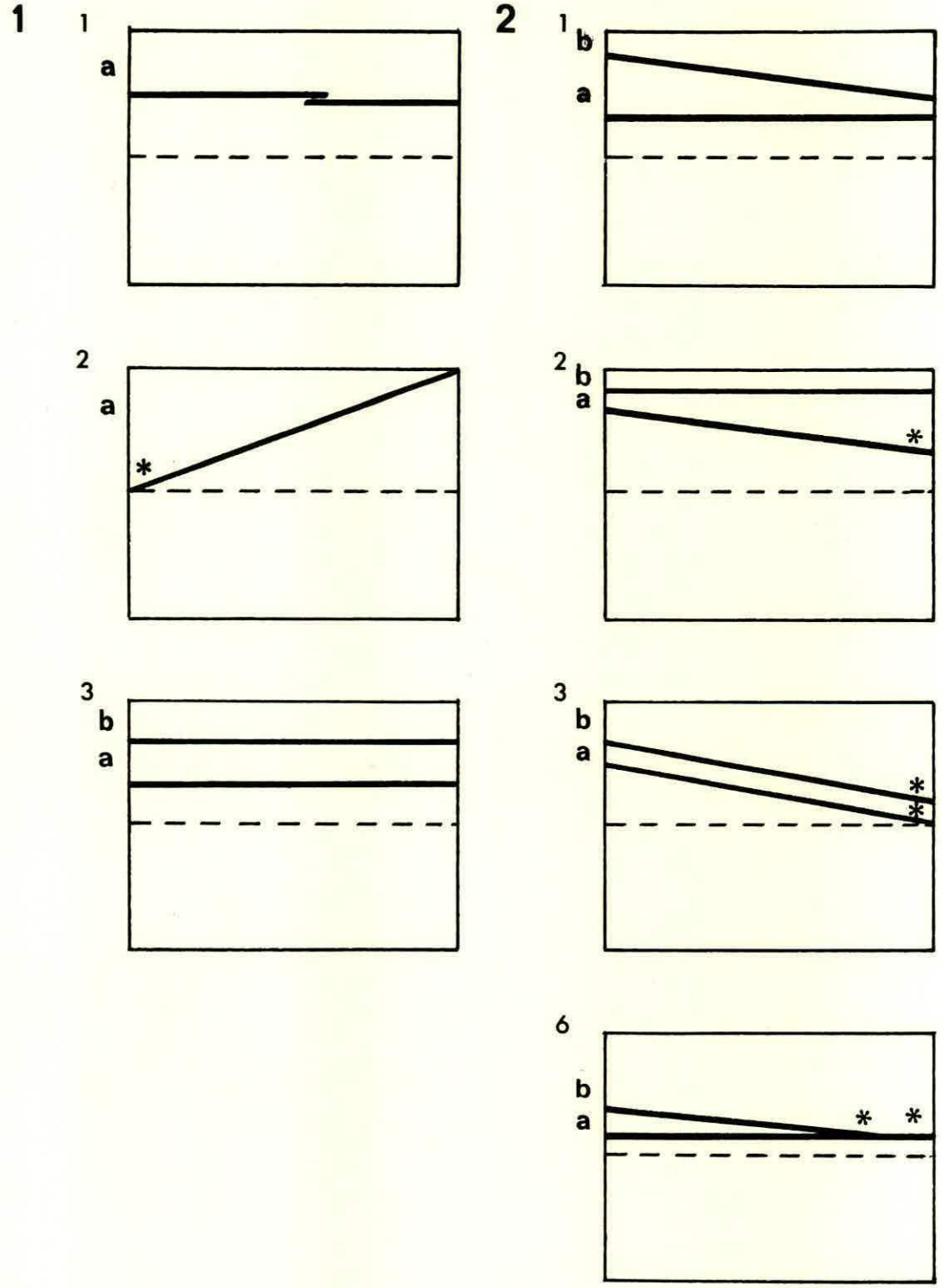


Fig. 4.7 (continued over)

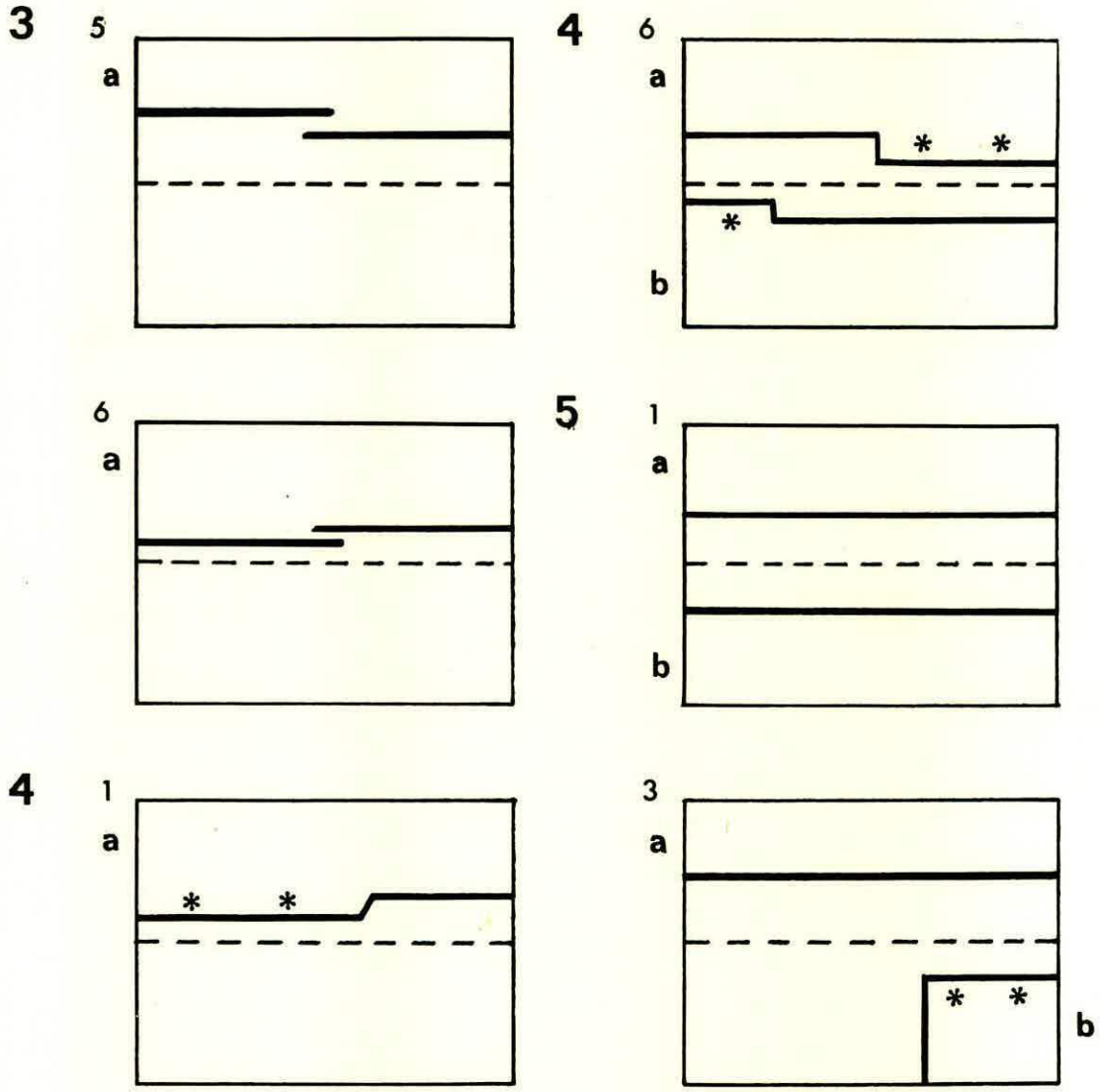


Fig. 4.7 Diagrams of cuts made in specimens described in the text.

Large nos: experiments. Small nos: specimens. ---- midline. Heavy lines: cuts. a and b: pieces referred to in Table 4.1. * position of first primordia to form in lateral, severed pieces where a complete row did not form simultaneously along the lateral edge of the cut.

TABLE 4.1 Time of Formation of Primordia Lateral to an Antero-Posterior Cut, Compared with the Time of Formation of Primordia in the Remainder of the Pteryla.

(Specimens and Pieces refer to Fig. 4.7 in which the cutting diagram for each specimen is shown.)

Experiment	Specimen	Piece of the Pteryla	No. of Rows of Primordia*		
			U	M	L
1	1	a	4	3	1
	2	a	2	0-2	1
	3	a	3	2	1
		b			F
2	1	a	2	2	1
		b			F
	2	a	3	2	1
		b			F
	3	a	2	0-2	1
		b	3	0-3	1
	6	a	2	1	1
		b	2	1	1
3	5	a	3	2	1
	6	a	2	1	1
4	1	a	2	1-2	1
	6	a	-	1-2	2
		b	-	1-2	2
5	1	a	-	2	1
		b	-	2	1
	3	a	3	2	1
		b	-	2	1

* Skin was observed at intervals in culture: the number of primordia in each piece was recorded at the time when primordia were first observed in the lateral piece.

Abbreviations:
- U Uncut, control side of the pteryla (excluding the midline).
- M Medial to the cut (excluding the midline): in all cases, primordia formed up to the cut before any formed in the lateral piece.
- L Lateral to the cut.
- F Failed to develop primordia; regressed in culture.

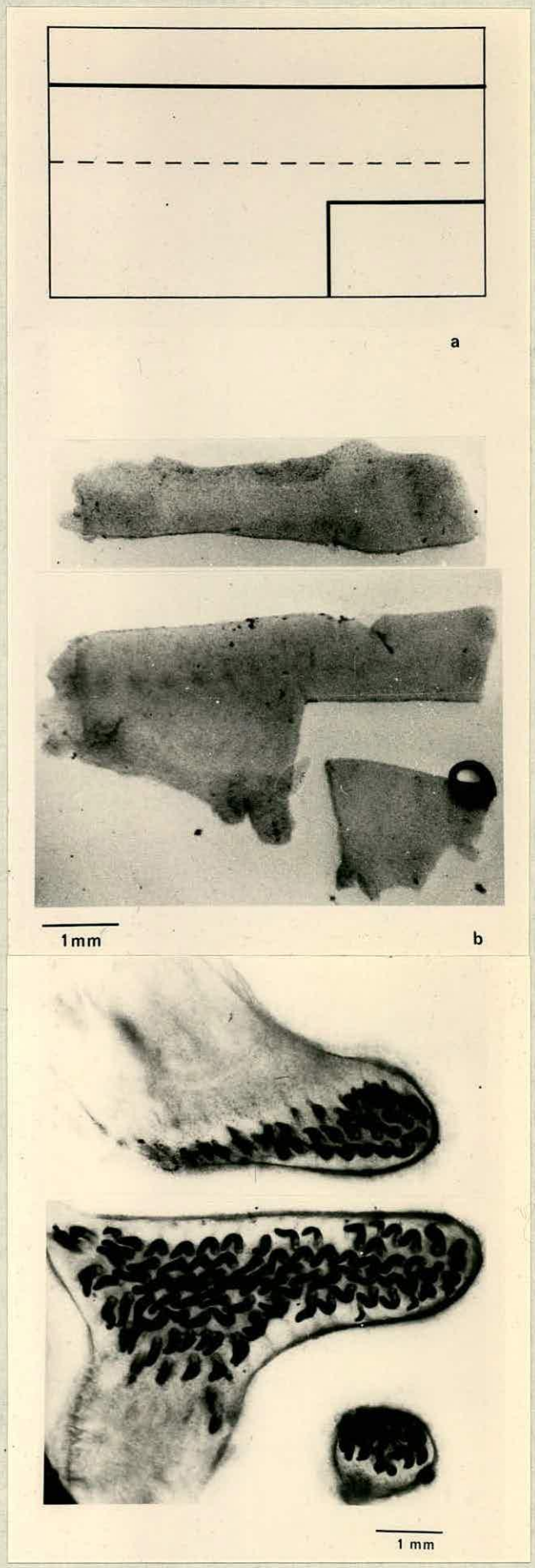


Fig. 4.8 (see over)

In all 4 cases, where a cut was made at a distance equivalent to about 1 to 2 rows from the lateral margin of the pteryla, the severed lateral piece degenerated without forming primordia. In all these cases a second cut was made closer to the midline and primordia developed in the piece of skin between the two cuts.

Periodic examination of the specimens developing in culture showed that, in every case, the first primordia to form in each severed lateral piece developed concurrently with primordia in an equivalent position on the uncut side of the pteryla and on schedule relative to primordia developing immediately medial to the cut (Fig. 4.7; Table 4.1). The time of formation of primordia within a row depended locally on distance from the midline, in the same way as on the uncut, control side of the pteryla. This was particularly clear in specimens which had been cut at an angle to the midline or where a stepped cut had been made. In the former case, the first primordia formed in a short, anterio-posterior row in the most medial region of the severed piece (Fig. 4.7). In the latter case, primordia developed on the medial side of the step before those on the lateral side of the step (Fig. 4.9). The remaining

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Fig. 4.8 The development of primordia in skin cut parallel to the midline and cut medio-laterally and anterio-posteriorly to isolate a small piece of the lateral part of the pteryla.
Feather filaments develop in each piece. a. cutting diagram, - - - - midline, heavy lines represent cuts; b. Stage 39+ cut specimen; c. 10 days after cutting.

Fig. 4.9 Pattern development over stepped anterio-posterior cuts. Tracing from photographs and microscopy notes of specimen 203/6. The specimen was cut as shown in a.
b. immediately after cutting; c. 23 hours; d. 42 hours. (- - - - midline, heavy lines = cuts; arrows = corresponding points; O formed primordium; ◌ just-visible primordium). Note that, lateral to the cut, primordia form first on the side of the step which contains the most medial skin. (see over)

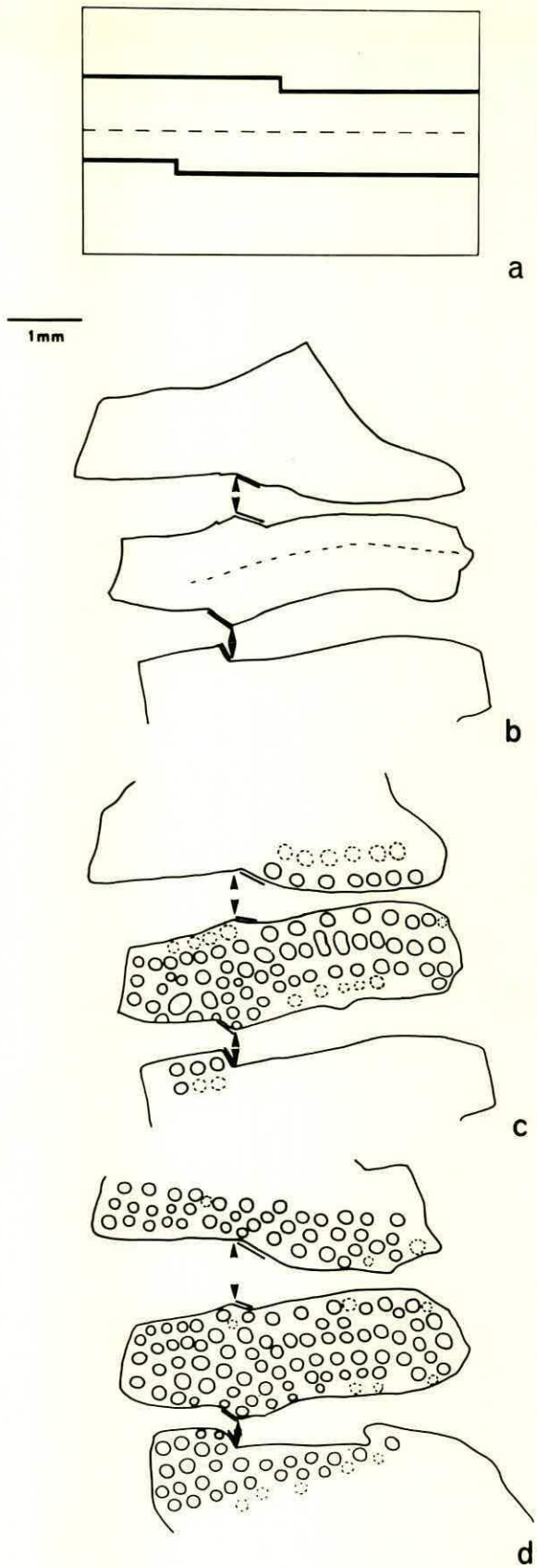


Fig. 4.9

rows in each severed piece developed in the same sequence, position, and orientation as those in equivalent parts of the uncut side of the pteryla.

Two further observations were made. First, in some cases, very small primordia were formed close to the cut, in positions expected relative to primordia in the preceeding row. These small primordia were more frequently observed on the medial, than on the lateral, side of the cut.

Second, in 3 specimens cut so that the lateral region remained connected to the main part of the pteryla by a short, narrow isthmus (Fig. 4.7, Nos. 1/1, 3/5 and 3/6) the initial alignment between the two pieces was preserved. In 2 of these cases, the lateral and medial pieces fused across the cut during culture, and were thus uninterpretable. In the remaining case (Fig. 4.7, No. 1/1), the pieces remained separated and the position of primordia, when they first became visible, lay precisely on the extensions of the chevron curves extrapolated from the pattern medial to the cut. Later, the lateral skin shrank anterio-posteriorly relative to skin medial to the cut and the register between the primordia was lost. (In all cases where lateral and medial skin was severed completely, the pieces shrank to different extents and any possible register between them was lost: see, for example, Fig. 4.8).

4. DISCUSSION

4.1 The Relation of Dermal Cell Patterns to the Emerging Feather Pattern

To establish whether, or not, intersecting arrays of dermal cells could play a part in initiating the morphogenesis of dermal condensations below established placodes or in determining the

feather pattern, serial sections parallel to the surface of the skin have been examined. The observation that arrays of dermal cells intersect at presumptive sites of condensation, while, in no case, were condensations present without associated arrays, implies that intersection of arrays preceeds condensation formation. This allows the possibility that arrays play a necessary rôle in the initial development of the condensation.

However, two observations suggest that it is unlikely that intersecting arrays determine the site of the new primordium. First, in skin with 5 to 7 rows of primordia arrays were only found to intersect in a minority of cases at the presumptive site of condensation one row in advance of formed condensations. Second, the present observations on transverse sections and the more definitive work already done by Sengel and Rusaouën (1969) indicate that unassociated placodes are present one row in advance of primordia in the majority of specimens at the same stage. Taken together these results indicate that placodes and intersections develop almost simultaneously at the primordium site and, indeed, suggest that placodes may develop before well-formed arrays intersect.

One could, of course, argue that intersection of matrix fibres or fine strands of aligned cells may slightly preceed placode formation and be sufficient to define the primordium site. This possibility is examined in the experiments described in Section 3.2 which critically test the hypothesis that lateral extension of a diagonal lattice-work of cells or fibres determines the feather pattern (Stuart et al, 1972).

Normal feather patterns form in skin which has been cut diagonally at a stage when no morphological patterns are detectable in lateral dermis. This result clearly does not match the crucial

prediction of the model of Stuart et al (1972). The implication is that the diagonal extension of cellular arrays from adjacent primordia in the preceeding row is not necessary to provide the activation to form primordia and does not play a primary rôle in defining their position.

Two alternative interpretations of this result are considered unlikely. First, an undetected diagonal lattice of fibres may already be present in lateral dermis at the time of cutting, which may define the feather pattern. Despite several attempts to detect such a lattice, (Wessells and Evans, 1968); Overton and Collins, 1976) there is currently no evidence that it exists. However, even if such a lattice does govern the pattern it must be present at or before the time when primordium morphogenesis begins in the pteryla: the question arises, what determines the pattern of the lattice?

Second, it is possible that anterio-posterior arrays of cells similar to those observed in some cultured specimens (Chapter 3) play a part in defining the position of primordia along the cut edge. However, this seems unlikely, for this organisation was a prominent feature in only a minority of specimens examined in Chapter 3, while normal patterns were formed in a great majority of specimens examined in the present experiments. Moreover, the morphology of these anterio-posteriorly oriented 'arrays' does not resemble normal diagonalintercondensation arrays, and appears too crudely organised to exert precise control over the position of new primordia.

It remains possible, of course, that arrays are formed in the normal position immediately lateral to the cut and that these play a part in the morphogenesis of primordia. However, in this

case, the position of primordia and of arrays cannot be determined by the hypothesised mechanism.

We may conclude that neither the initiation of primordium development nor the definition of the positions of new primordia are governed primarily by the progressive lateral organisations of arrays of cells from existing areas of pattern immediately in advance of primordium morphogenesis.

4.2 The Initiation of Development in Successive Rows of Primordia

The general nature of the process which does govern the initiation of primordium development has been investigated in the experiments described in Section 3.2. Specifically, these experiments test the hypothesis that the formation of successive rows of primordia depends on the propagation of an activated or competent state across the skin immediately in advance of primordium formation.

The results indicate that such a propagation mechanism is not an absolute requirement for the formation of successive rows: primordia develop lateral to an anterio-posterior cut made in the skin at the line, or one-row stage. Since the same results were obtained when pieces of the pteryla were cultured separately, it is clear that no necessary impulse passes across the cut (for example, by diffusion) in these experiments. In these respects, the present results confirm the conclusions of Linsenmayer (1972) which were based on experiments with thigh skin cultured on the CAM.

The results further strongly suggest that the row immediately lateral to an anterio-posterior cut is initiated by the same mechanism as operates in an equivalent region of uncut pteryla. This conclusion is based on the observation that the time at which primordia develop lateral to the cut depends strictly and locally on

their original distance from the midline and corresponds, within the limits of error in the present analysis, to the time at which primordia form in an equivalent region of uncut pteryla in the same specimen. It is very unlikely that these conditions could arise by global regulation lateral to the cut leading to the establishment of a new initial row from which propagation was resumed.

Taken together, these conclusions strongly suggest that, during normal development, the impulse to form primordia is not propagated across the pteryla immediately in advance of primordium morphogenesis (Ede, 1972; Novel, 1973; Stuart et al, 1972). Similar reasoning, applied to pieces of skin isolated by partial anterio-posterior and medio-lateral cuts (Fig. 4.8), implies that there is no essential activation impulse propagated posteriorly, in the region examined, close to the time of primordium formation (Ede, 1972).

The development of the last one or two rows of primordia which are formed in the pteryla in culture must be exempt from these conclusions till more information is available, since this region consistently failed to develop primordia when severed from more medial skin. It is possible that these lateral pieces are merely too narrow to maintain their integrity in culture. An alternative possibility, which remains to be tested, is that the intrinsic capacity for feather development has not yet been established in this region by the time the initial row is about to form: the degeneration of these extreme lateral pieces may occur for the same reason as whole skin younger than $5\frac{3}{4}$ days degenerates in culture. For example, the determination to form feathers may be propagated laterally, beginning at the midline at $5\frac{3}{4}$ days and reaching extreme lateral regions more than 12 to 18 hours later: that is, after the

time of cutting. This could be further examined by cutting skin in this region at Stage 30 to 31 and observing the development of the severed piece.

These conclusions have an important implication. The time at which primordia will form over most of the pteryla is independent of the continuity of the skin and must therefore be set locally before the beginning of morphogenesis. Since this temporal organisation is dependent on position, it may be termed a 'time map' for the onset of morphogenesis. The nature of this map is discussed in Chapter 6.

4.3 The Positions of New Primordia

The pre-existence of temporal organisation emphasises that the skin is not entirely unpatterned with respect to primordium initiation. With regard to the positions of new primordia, several observations bear on the problem of how far these are determined before morphogenesis begins and how far the spatial pattern is generated as successive rows form.

The most important of these observations is that both in vivo and in culture the positions of primordia in the spinal pteryla fall on curved chevrons as the pattern emerges. The patterns formed in a small sample of explants from the cervical tracts indicates that the same is true in this region. Linsenmayer (1972) has also noted that primordia lie on curved chevrons during the development of thigh skin explanted before primordia form and cultured on the CAM. Although the curves observed in freshly excised skin may derive in part from distortion due to mounting, the curves observed in skin developing in culture cannot be the result of distortion of an already formed pattern of primordia. The

evidence indicates that chevrons do not become curved through differential growth after morphogenesis has established a linear diamond pattern (Sengel, 1976b), since the chevron curves remain approximately constant after the pattern has emerged. Moreover, both in the present culture system and on the CAM the skin does not grow as it does in vivo (see Chapter 3 and Linsenmayer, 1972), but the curvature of chevrons resembles that found in skin from 10 to 12 day-old embryos. The consistent relation of new primordia to existing chevron curves, and the observation that, at least in some cases, newly formed primordia were not equidistant from their neighbours suggests that primordia are not positioned by a simple packing constraint such as is proposed in current models (Ede, 1972; Novel, 1973). It is possible that more complex pattern-forming rules might enable these models, or other models based purely on a template mechanism, to account for the curvature of chevrons by generating successive slight changes in the angle between existing and incipient primordia. However, it is difficult to see how such a mechanism could form different chevron curves over different parts of the skin. A simpler alternative is that organisation which governs the general direction or the position of chevrons is established in a linear, medio-lateral direction some time before morphogenesis and becomes curved as a result of skin growth before morphogenesis begins. Since, at the time of morphogenesis in lateral skin, the chevron configuration is quite close to that found at 10 days or even in the adult, it seems likely that such growth does not occur during the period immediately surrounding morphogenesis, but may occur some time earlier. The observation that, in a single specimen, the position of primordia on the lateral side of an anterio-posterior cut coincided with the extrapolated medial chevron

curves points tentatively to the possibility that the position of individual chevrons may be established in lateral skin before the cut is made. Clearly, more experiments of this type are needed before this point can be established. With respect to the kind of organisation which could be involved in influencing the chevron positions, it would be interesting to look more closely at the structure of the broad, low ridges observed in freshly excised specimens. The possibility that the number and position of chevrons may be related to pre-existing embryonic structures is discussed further in Chapter 6 in relation to the literature on the patterns of Reptilian scales.

Despite the possibility that the positions of chevrons are determined before morphogenesis begins, the positions of individual primordia can be influenced close to the time of morphogenesis by limiting the area available or by distortion of the whole skin. Two observations point to this conclusion.

First, 10 to 12 rows form across the pteryla in culture whereas 17 to 19 rows form in vivo where the skin grows. Since the spacing in culture and in vivo is regular, it follows that the rows which form in culture do not form in pre-established positions while the intervening sites are suppressed. These observations confirm the conclusion of Linsenmayer (1972) and Novel (1973) with regard to the periodicity in one dimension, although the possibility that a previously defined pattern is changed as a regulative response to culture without lateral growth still cannot be ruled out.

Second, anterio-posterior positions of chevrons can be disrupted in the anterior region - even in the initial rows - as a result of explantation and culture. Although this conclusion is complicated by the fact that in vivo the pattern in this region is

not always entirely regular, it is clear that some displacement of primordia from the normal pattern does occur in culture. This may be due, in part, to the anterior region being less advanced developmentally (as far as can be judged by morphological development of primordia) than the posterior region at the time of explantation: anomalies are not so common in the anterior-region of skin explanted when a few rows are already present. The fact that the degree of medial development influences the regularity of the lateral pattern formed in culture may derive from the operation of a template mechanism of pattern formation which is given stability by the existing medial pattern, or may merely be a consequence of a more mature developmental state in the lateral skin of these specimens. Posteriorly, the anomalies which occur in the midline are almost always regular and symmetrical and, since similar anomalies are found in vivo with roughly the same frequency it is probable that they are not the result of culture.

The observation that displacement of primordia appears, in some cases, to correlate with distortion of the available space, suggests that local constraints do play a part in governing the positions of primordia. Observations on the way the pattern can be disturbed relate to the nature of these constraints.

Fusion of primordia at adjacent sites suggests that simple steric inhibitory interactions are not operative between primordia at the earliest stages of morphogenesis (Novel, 1973). Fusions are also difficult to account for in simple terms by the model of Ede (1972). Indeed, the reason for the occurrence of dual primordia is not clear. Similar dual primordia were observed by Hamilton (1965) in skin treated with berylium and manganese in combination, during experiments to test the importance of alkaline

phosphatase in feather development. However, in the present experiments they seem to arise, at least in some cases, by physical compression of the skin. It is possible that fusions between adjacent sites in a latent pattern occur before the onset of morphogenesis, but this is difficult to reconcile with the evidence that the medio-lateral positions of primordia are not irreversibly fixed at this time. The time when physical distortion of morphologically unpatterned skin can influence the position of primordia must be examined in more detail in order to assess the possibility that already-defined sites can be fused before morphogenesis begins.

One further observation is relevant to how the sites of primordia are established: this is the fact that small, circular primordia are formed close to an anterio-posterior cut. A similar phenomenon was observed Koning and Hamilton (1954). Since these small primordia were neither touching adjacent ones nor bisected by the cut, it seems that the position of the centre of the primordium is determined first (possibly, though not necessarily before the skin is cut) and that the diameter is governed, secondarily, by the available space. The fact that, in most cases, these small primordia lay medial to the cut suggests that their diameter is constrained by the proximity of more mature primordia. It is possible that, given the spacing between primordium centres, the diameter of primordia in the normal pattern is governed in this way.

In conclusion, the positions of individual primordia are not irreversibly established till close to the time of morphogenesis. However, although available space may constrain the positions of primordia, the evidence suggests that their positions are not established by a simple packing mechanism acting in otherwise

unpatterned skin, and points to the possibility that the positions of chevrons may be determined by organisation existing in the skin some time before morphogenesis begins.